THE ROLE OF THE GUT MICROBIOTA IN COLONIZATION RESISTANCE AGAINST *CLOSTRIDIUM DIFFICILE*

by

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The world is magic.
Science is but an insipid style of sorcery.
-- Rudy Rucker

DEDICATION

I would like to dedicate this dissertation to my parents, Rita and Paul Schubert. Thank you for always being supportive of me in the best and worst of times. If it were not for your example, encouragement, and love, I would not be where I am today.

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ABSTRACT

The human body is host to a complex community of microorganisms collectively known as the microbiota. Specifically, the gastrointestinal tract harbors trillions of bacteria with important roles in host health. The gut microbiota can play a critical role in preventing invading microbes from persistently colonizing the gastrointestinal tract, a phenomenon known as colonization resistance. Perturbations, such as antibiotic treatments, which change the structure of the gut microbiota, may in turn alter colonization resistance to pathogenic microbes. Epidemiological studies demonstrate that antibiotic use is a major risk factor for acquiring a *Clostridium difficile* infection (CDI), which is the leading nosocomial infection in the United States. I hypothesized that multiple bacterial populations contribute to the inhibition of *C. difficile* colonization. To test this hypothesis, I investigated the role of the gut microbiota in colonization resistance against *C. difficile* in both humans and mice. Across both hosts, I demonstrated that a loss in Lachnospiraceae, Ruminococcaceae, Porphyromonadaceae, *Bacteroides*, and *Alistipes* were highly correlated with loss of

colonization resistance. Using mathematical modeling of microbiota abundance data, I showed that the microbiota was a powerful indicator of disease state and could be used to accurately predict levels of *C. difficile* colonization, effectively measuring the microbiota's level of resistance against this pathogen. My results demonstrated the importance of the microbiota as a whole in conferring colonization resistance. These studies provide important insight into the relationships between members of the microbiota and *C. difficile* and further define a consortia of bacteria from which efficient probiotic therapies can be designed.

Chapter I

Introduction

The Human Microbiota

The human body is host to a diverse set of microorganisms, comprised of bacteria, viruses, protozoa, fungi, and eukaryotes, known as the microbiota. The most widely studied of these microorganisms are bacteria, which outnumber our own cells 10-fold (1) and collectively have over 100 times the number of human genes (2). The majority of these bacteria reside throughout the gastrointestinal (GI) tract, reaching upwards of 100 trillion bacteria cells (3). These bacteria have adapted their lifestyle to the gut environment. The structure of these communities, referring to which bacterial species are present and their abundance, develops with its human host beginning at birth (4-10). The intimate relationship between humans and their associated microbes has made the microbiota an integral part in normal host function.

Defining the "Healthy" Microbiota

Previously, characterization of the microbiota was limited because the laboratory growth conditions for most gut bacteria are yet unknown, and consequently only a

subset of the microbiota could be characterized. With the advent of next-generation sequencing came the ability not only to uncover uncultivable bacteria but also the structure of the microbiota in a massively parallelized manner. Huge efforts have been taken to define the “healthy” microbiota structure both in humans (11-16) and in mice (17-19), which are often used for *in vivo* models. These studies are important in providing a baseline understanding of the microbiota in a healthy state. Cataloguing the healthy human gut microbiota has revealed that there is no “core” bacterial community defined by a subset of bacterial members (20). In a cohort of 154 individuals there was no bacterial species that could be found in the microbiota of every subject, underlining a high level of inter-individual taxonomic diversity across the human population (20). There are, however, proposed “core” functions represented by the microbiota (15, 20), which have been described through metagenomic sequencing of the human gut (21). Three broader gut “community types” called enterotypes have been described to categorize the microbiotas of healthy individuals (16). These enterotypes are defined by a predominance of *Bacteroides*, *Prevotella*, or *Ruminococcus* (16). These community types are likely not discrete groupings but rather exist on a gradient. Using multiple mathematical approaches to define these microbiota types, continuous gradients of bacterial abundances have been observed within body sites as well as bi- and multi-modal abundance distributions (22). Another study of community types across human body sites has shown that community types are distinguishable not simply by the most predominant bacterial population, but rather by more complex assemblages of bacteria likely shaped by overall life history factors (11). In the human gut microbiota, the Bacteroidetes and Firmicutes bacterial phyla dominate overall with a higher proportion

of Bacteroidetes (19, 23). This is also similar to what is observed in mice. Comparison of the human and mouse gut microbiota at higher taxonomic levels shows strong overlap in community structure. Major differences between these hosts are observed at the genus level, where only 15% of mouse genera were also found in humans (19). Thus, the definition of a “healthy microbiota” covers a wider scope of community structures. Broadly we can discern healthy patterns across the human population, but at refined taxonomic levels, healthy is best assessed on an individual basis. These observations are important considerations when studying the microbiota during disease states and developing microbiota-based therapies.

The Gastrointestinal Environment

Not only are there inter-individual differences found in humans, but there is also intra-individual variation in the structure of the microbiota across and even within body sites (14). The gastrointestingal tract, comprised of the stomach, small intestine, cecum, appendix (in humans), large intestine, and rectum, represents diverse environments, along which are several environmental changes (**Figure 1.1**). Microorganisms, including external pathogens, entering the body orally and traveling through the gastrointestinal tract must be able to survive these extreme environmental changes. To name a few, pH and oxygen gradients exist along the gut, starting with the acidic and aerobic stomach to the more neutral and anaerobic environment of the colon. Additionally there are shifts in general nutrient availability as the host and resident microbes break down diet components. For example, limited levels of short chain fatty acids (SCFAs) are observed in the small intestine, which increases closer towards the colon (24-26).

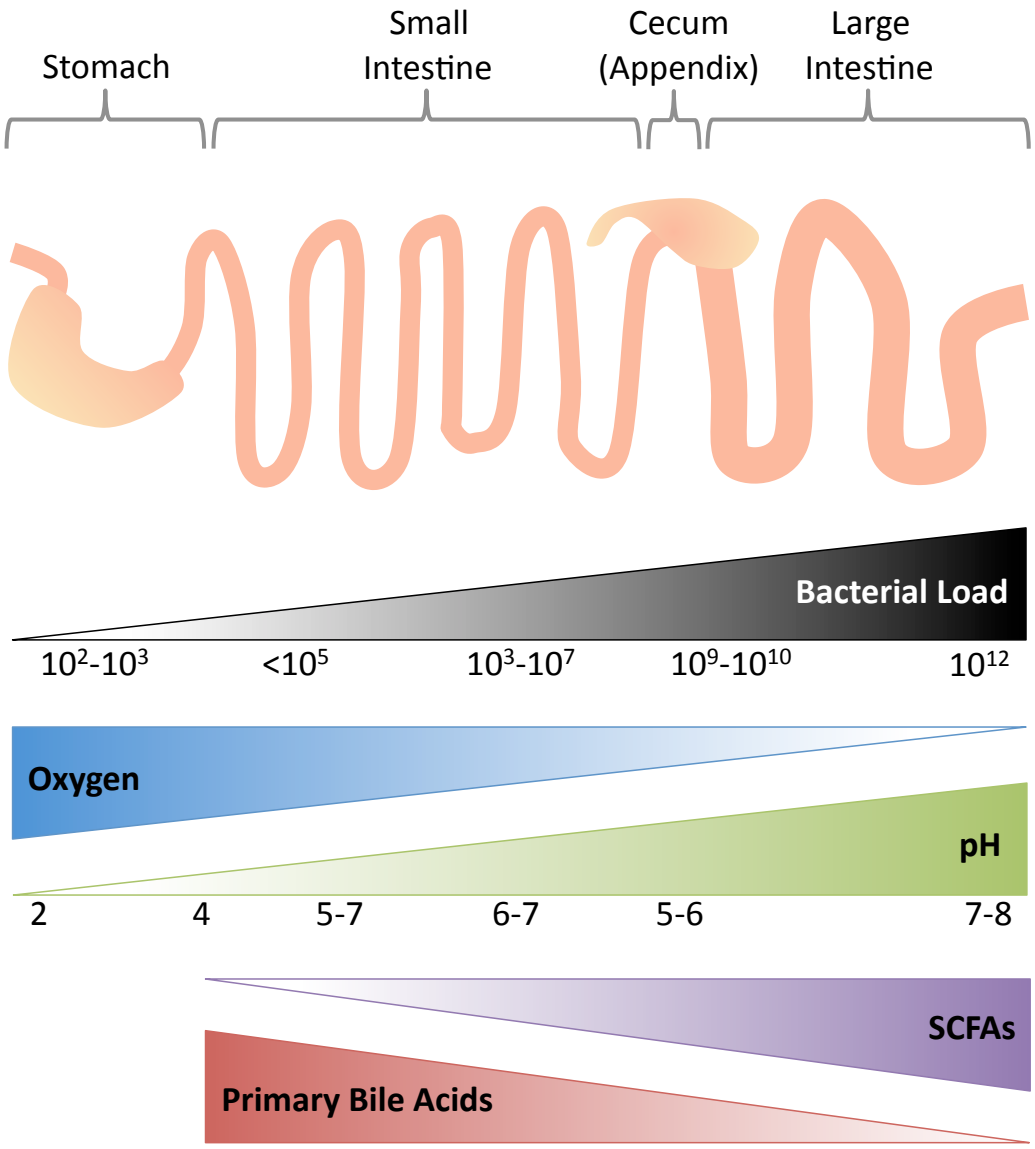

FIGURE 1.1 Environmental changes throughout the gastrointestinal tract
The specific ranges of bacterial load and pH levels throughout the GI tract are shown (31). In mice, the GI tract includes a functioning cecum. While in humans, the cecum represents the transition from the small to large intestine. Humans additionally have an appendix, which has no known function, but may serve as a reservoir for resident bacteria.

Image adapted from Mowat A.M. and Agace W.W. 2014. Nature. Rev. Immunol. 14:667, See ref (32)

Consequently, these unique environments along the gastrointestinal tract harbor different amounts of total bacteria with varying compositions. Within the colon exist further microenvironments. The lumen of the colon is anaerobic. Closer to the epithelial layer there is a gradual increase in oxygen creating a microoxic environment (27). This radial oxygen gradient contributes to the segregation of oxygen tolerant and intolerant bacteria (27). An outer and largely sterile inner layer of host mucus protects the epithelium acting as a physical barrier to prevent bacterial proximity and translocation (28-30). These diverse conditions create a number of habitats in the gut for bacteria to reside and also represent environmental challenges for orally ingested microorganisms traveling through the gut.

The Microbiota's Dual Role in Human Health and Disease

Many of the resident microbiota have a mutualistic relationship with their host, meaning that both the microbiota and the host benefit from this relationship. In fact, the microbiota has essential roles in human health. Specifically within the gut, the microbiota functions in digestion of host-indigestible carbohydrates (2, 3), synthesis of vitamins (2), maturation of the immune system (33, 34), regulation of immune homeostasis (34-36), and defense against invasive pathogens (37-40). When the microbiota is significantly perturbed, it can result in a state of dysbiosis, which describes a destabilized deviation of the bacterial community's structure from its healthy baseline. Dysbiosis has been implicated in a number of diseases including inflammatory bowel disease (41-44), metabolic disorders (45, 46), obesity (19, 47), colorectal cancer (48-

51), and several gastrointestinal infections (52-56). Thus, maintaining a diverse, stable microbiota is critical for human health.

The Microbiota Confers Colonization Resistance

Of the many beneficial roles of a healthy gut microbiota, the ability by the indigenous microbiota to protect against invasive pathogens from persistently colonizing the gastrointestinal tract is called colonization resistance. The microbiota is known to play a significant role in colonization resistance against several GI bacterial pathogens, including VRE (40, 57-60), *Salmonella enterica* serovar Typhimurium (54, 61-66), *Escherichia coli* (67-75), and *Clostridium difficile* (52, 58, 76-80). In the case of *C. difficile* infections, epidemiological data has shown that antibiotic use is one of the major risk factors (81-86). Antibiotics alter the normal bacterial structure of the microbiota towards a state of dysbiosis. In this state, the host is susceptible to colonization and infection by *C. difficile*. It is unknown what precise changes to the microbiota structure and function are responsible for the loss in colonization resistance against *C. difficile*.

Clostridium difficile Infections

Epidemiology

C. difficile is an emerging pathogen in the United States, recently surpassing MRSA as the most reported nosocomial infection. It is estimated there were 450,000 *C. difficile* infections (CDI) and 29,000 related deaths in 2011 (87). As a result, the associated health care costs are estimated in excess of $1 billion annually. Individuals colonized with *C. difficile* may be asymptomatic (approximately 3-5% of healthy adult

population) or experience a range of CDI severity from mild and moderate disease with diarrhea and pseudomembrane colitis to severe infection with toxic megacolon and sometimes death (81). Epidemiological studies identify antibiotic use, age over 65, prolonged stay at a health care-associated setting, comorbidities, immunocompromised state, and proton pump inhibitors as major risk factors for CDI (81-85, 88-90). Interestingly, many of these risk factors also have effects on the stability and structure of the microbiota (88, 89, 91, 92). Furthermore, in the environment *C. difficile* exists in its highly resistant spore form, which makes it difficult to eradicate. This is highly problematic in health care-associated settings, where an individual may be exposed to several CDI risk factors or perturbations to the microbiota and where subsequently an estimated 66% of all CDI-onset occurs (87).

C. difficile *Colonization and Infection*

The dynamics of *C. difficile* colonization and infection is an area of great interest. When the transmissive spore form of *C. difficile* has been orally ingested, spores safely travel through the acidic stomach environment to the small intestine (**Figure 1.1**). Here germination factors, including primary bile acids such as taurocholate and the amino acid glycine, induce germination of spores to vegetative cells (79, 93). These vegetative cells travel to the cecum (of mice) and colon in search of resources for growth. In a non-susceptible state containing an intact microbiota, *C. difficile* is outcompeted for these resources. In a susceptible state containing a dysbiotic microbiota these resources are more readily available (65, 94). The earliest observations of CDI at 6 hours post infection reveal *C. difficile* vegetative cells in the cecum of cefoperazone-treated (79).

Approximately 24 hours later *C. difficile* spores are largely detectable in the cecum, colon, and stool. This increase in spore production towards the distal gastrointestinal tract could be important for transmission of *C. difficile.* Sporulation coincides with production of its two main toxins, the enterotoxin A and the cytotoxin B (79). These glucosylating toxins disrupt Rho family GTPases, causing actin depolymerization, loss of cell architecture, cell death, and breakdown of epithelial barrier function (95). There is a third known toxin, the cytolethal distending toxin or binary toxin (CDTa and CDTb), whose role in the pathogenesis of CDI is unclear. Epidemiological studies suggest that it enhances pathogenesis, as humans infected with a CDT carrying strain have a 60% increase in fatality (96). These toxins increase gut inflammation and induce the histopathological changes to the large intestine.

Current Treatment Courses

Standard treatment for CDI includes antibiotic regimens of metronidazole or vancomycin, which inhibit *C. difficile* but also continue to perturb the microbiota structure. In as many as 25-30% of cases, individuals will have recurrent CDI following clearance of initial infection (97-100). Treatments for CDI clearly require consideration of its effect on the microbiota and should aim to restore an overall healthy microbiota structure. In cases of recurrent CDI, fecal microbiota treatment (FMT) has been implemented with increasing use. This requires the collection of stool sample from a healthy donor, preferably a significant other or maternal-line first degree relative, who are likely to harbor more similar microbiota structures (101, 102). Stool is screened for a variety of pathogens (101) and prepared for transplant into the recipient via enema,

colonoscopy, or nasogastric tube. Following successful transmission of the donor bacterial community, the microbiota of the recipient shifts towards a structure more similar to the donor's (103, 104). In a microbiota survey of 14 individuals before and after receiving FMT, the level of Bacteroidetes increased, Proteobacteria decreased, and diversity increased (103), which are all characteristic of "healthy" human gut microbiotas. A systematic review of FMT for CDI reported that the efficacy of FMT is 90% (105). The success of this treatment emphasizes the importance of the microbiota as a whole in protection from *C. difficile.* That we do not fully understand the mechanism behind this treatment highlights the need for a greater understanding of the interactions between the microbiota and *C. difficile*.

The Microbiota and *C. difficile*

Previous work characterizing the microbiota of humans and mice in the context of CDI provide a glimpse into potentially protective and alternatively susceptible community structures (**Table 1.1**). In humans, fecal samples taken from patients who subsequently developed CDI have been compared to age and sex matched controls (106). The microbiota of patients with CDI have increased levels of Enterococcaceae and decreased Clostridiales Incertae Sedis XI and Bacteroidaceae compared with the matched controls. Additionally, decreased diversity is seen compared with controls. Individuals with recurrent CDI have an even greater drop in the diversity of their microbiota compared to individuals with an initial CDI episode (107).

Bacteria Associated with Resistance or Susceptibility to CDI			
Bacteria	**Host**	**Potential Role**	**References**
Bacteroidaceae	Human, Mouse	Resistance	18, 78
Bacteroidaceae *(Bacteroides thetaiotaomicron)*	Mouse	Susceptibility	65
Porphyromonadaceae, e.g. *Barnesiella*	Human, Mouse	Resistance	18, 76, 78
Lachnospiraceae, e.g. *Blautia*, *Clostridium clostridiaforme, Clostridium scindens*	Human, Mouse	Resistance	18, 52, 76-78, 116, 143
Ruminococcaceae	Mouse	Resistance	78
Clostridiales Incertae Sedis XI	Human	Resistance	18
Lactobacillaceae	Mouse	Susceptibility	52, 78, 80
Enterococcaceae	Human	Susceptibility	18, 143
Proteobacteria	Human, Mouse	Susceptibility	52, 76-78
Enterobacteriaceae	Mouse	Susceptibility	52, 76-78
Pseudomonadaceae	Mouse	Susceptibility	52
Verrucomicrobiaceae *(Akkermansia)*	Mouse	Susceptibility	78
Verrucomicrobiaceae *(Akkermansia)*	Mouse	Resistance	143
Erysipelotrichaceae *(Coprobacillus)*	Mouse	Resistance (indirect)	143

TABLE 1.1 Bacteria associated with resistance or susceptibility to CDI

These human studies however lack the baseline healthy state of the microbiota of these individuals. Murine models of CDI have been utilized because we can control for specific changes that occur before and after antibiotic perturbations and the introduction of *C. difficile.* The first model of CDI in conventional mice that more closely mimicked human disease used a 5 antibiotic cocktail made of gentamicin, kanamycin, colistin, metronidazole, and vancomycin in water followed by a single intraperitoneal injection of clindamycin prior to *C. difficile* challenge (108). When challenged with *C. difficile* vegetative cells, mice either displayed severe signs of CDI or appeared healthy with no significant weight loss (52). The microbiota of healthier mice had a high abundance of Lachnospiraceae, similar to control mice without antibiotics, while sick mice contained mostly Enterobacteriaceae, similar to antibiotic treated mice.

Since the introduction of this initial mouse model, other antibiotic regimens have been used to assess how they alter the gut microbiota and ultimately colonization resistance. Across these different antibiotic perturbations, similar microbial patterns have been observed in both *C. difficile* susceptible and resistant communities. Ten days of cefoperazone (a third generation cephalosporin) in the drinking water with or without a follow-up clindamycin injection resulted in Lactobacillaceae dominance of the microbiota and susceptibility to *C. difficile.* A single clindamycin injection has also shown to induce susceptibility and a high prevalence of Enterobacteriaceae (109). Enterobacteriaceae along with Verrucomicrobiaceae were increased in the gut microbiota of mice treated with tigecycline, which *C. difficile* successfully colonized (78). Across these studies, comparison of *C. difficile* susceptible communities with untreated controls often correlates with a significant loss in Clostridiales (e.g. Lachnospiraceae

and Ruminococcaceae) and Bacteroidales with a loss in colonization resistance (**Table 1.1**). Direct evidence of a Lachnospiraceae isolate's contribution to resistance to *C. difficile* was further tested through monocolonization of germfree mice. These mice have increased resistance to *C. difficile*, compared with *E. coli* mono-colonization, which had no effect on *C. difficile* levels (77).

Mechanisms of *C. difficile* Colonization Resistance by the Microbiota

The mechanisms of colonization resistance by the gut microbiota against *C. difficile* are yet to be uncovered. Moreover it is not fully appreciated at what stages of *C. difficile* infection these protective functions act on. It is known that mice with an intact normal microbiota are resistant to *C. difficile* colonization. An examination of the murine gut metabolites before and after cefoperazone-induced susceptibility to *C. difficile*, as well as after challenge with *C. difficile*, highlighted an important role for the microbiota in limiting resources necessary for *C. difficile* spore germination and outgrowth (80). Following antibiotic perturbation, there is a shift from secondary to primary bile acids in the gut, specifically an increase in the availability of primary bile acid taurocholate, a germinant for *C. difficile* spores. This leads to the hypothesis that the gut microbiota can indirectly inhibit germination of *C. difficile* spores ingested by the host. However, recent e*x-vivo* and *in vivo* experiments show that *C. difficile* spores are able to germinate in the distal small intestine of untreated mice (79, 93). Thus additional mechanisms of colonization resistance are necessary for inhibition of subsequent stages of *C. difficile*'s colonization.

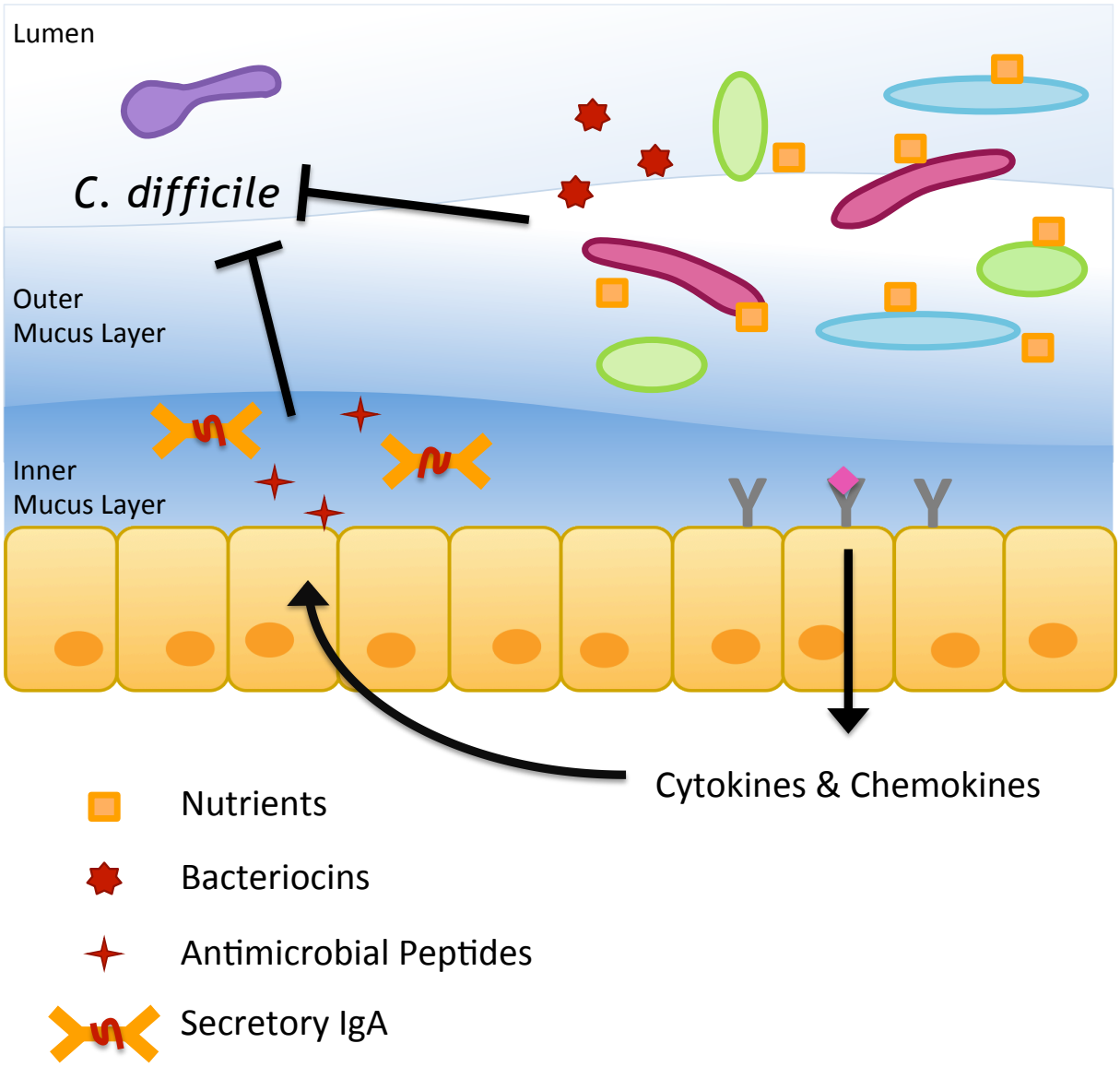

FIGURE 1.2 Mechanisms of colonization resistance by the microbiota against *C. difficile*

Competition for nutrients plays a significant role by limiting growth of vegetative *C. difficile* (**Figure 1.2**). Early studies in a continuous flow culture model of the cecal mouse microbiota have shown that *C. difficile* utilizes glucose, N-acetylglucosamine, and N-acetylneuraminic acid, although it does not contain the hydrolytic enzymes necessary to free these monosaccharides which are largely present in mucin (110). Furthermore, in the presence of the microbiota *C. difficile* is outcompeted for these resources and its growth is suppressed (65, 110). Sialic acids and succinate are two additional nutrient sources for *C. difficile*. In the presence of the indigenous microbiota, these resources are scarcely free in the milieu. However, following antibiotic treatment or polyethylene glycol treatment to significantly alter the microbiota, these nutrients are readily available to support *C. difficile*'s growth (65, 94). Depending on the environmental nutrients available to *C. difficile,* i.e., a polysaccharide rich versus deficient host diet, it can adapt by metabolizing succinate or sialic acids in host mucin, respectively (94). In a previous metabolomic survey, following cefoperazone treatment the levels of sugar alcohols and carbohydrates favorable for *C. difficile* outgrowth, including sorbitol, mannitol, raffinose, stachyose, and fructose also increased, creating an unclaimed pool of nutrients for *C. difficile*'s growth (80).

Microbiota products and metabolic byproducts have been shown to inhibit *C. difficile* growth. Several microbiota-produced bacteriocins in the gut have been demonstrated to have anti-*C. difficile* activity with minimal consequences to the overall structure of the microbiota. For example, indigenous *Bacillus thuringiensis* produces a bacteriocin named thuricin CD with a narrow target range that includes *C. difficile* (111).

R-type bacteriocins, similar to those found in *Pseudomonas aeruginosa*, similarly have anti-*C. difficile* activity (112). These can be modified to further increase specificity for fluoroquinolone-resistant BI/NAP1/027 strains of *C. difficile* (113), which have emerged in several outbreaks and have been attributed with more severe infection (114-116). Additionally, the baseline gut microbiota (prior to antibiotics) has high levels of deoxycholate (80, 117), which is a secondary bile acid made by microbiota members that inhibits *C. difficile* growth (118). For example, *Clostridium scindens*, a Lachnospiraceae, has enzymes that are able to biotransform cholate into deoxycholate, which inhibits *C. difficile* growth in a dose-dependent manner (117-119).

The Microbiota Stimulates Host Immune Defenses

The microbiota also indirectly inhibits *C. difficile*'s colonization and infection through stimulation of the immune system. Neutrophils are critical innate immune cells in early response to CDI (120, 121). Stimulation of NOD1 by *C. difficile* ligands mediates neutrophil recruitment through the production of the CXC-chemokine ligand 1 (CXCL1) (122). CXCL1 production by epithelial cells requires the secreted cytokine IL-1β, which is stimulated in part by the indigenous microbiota translocated across the epithelium during infection with toxigenic *C. difficile* (123). Innate immune activation via microbiota signaling through toll-like receptors (TLRs) and the MyD88 pathway is important for protection against severe CDI (120, 124). MyD88 deficient mice have a significant 1000-fold reduction in expression of colonic CXCL1 compared with wild-type (WT) mice (120). Subsequently, these mice also have significantly less neutrophil and monocyte recruitment to the colonic lamina propria.

The significance of microbiota stimulation through several TLRs has been examined. TLR5 stimulation by flagellin treatments (using *Salmonella enterica* serovar *Typhimurium*-derived flagellin) protected against mortality (125) using the Chen *et al.* CDI murine model (108). In the absence of TLR5, this protection was ablated. These flagellin treatments significantly reduced *C. difficile* levels and pathology in the cecum and colon, showing the potential influence of other microbiota members in stimulating the immune system to protect against CDI. TLR4 deficient mice have more severe disease than WT or TLR2 deficient mice (126) using the same model of CDI (108). That these TLRs have differential roles during CDI may not be surprising given their recognition of different bacterial cell wall components (127). Importantly, TLR4 deficient mice are not susceptible to CDI without prior antibiotic treatment (126), emphasizing the primary role of the microbiota in *C. difficile* defense.

Innate defenses against other gastrointestinal pathogens suggest potential mechanisms that are also active against *C. difficile.* Antimicrobial peptides (AMPs), such as RegIIIγ, are important in defense against several gastrointestinal pathogens including *Citrobacter rodentium* (128, 129), *Listeria monocytogenes* (130-132), *Salmonella enterica* serovar *Typhimurium* (129), *Staphylococcus aureus* (132), and Vancomycin-Resistant *Enterococci* (VRE) (133). Epithelial cells express these AMPs via microbiota signaling through MyD88 (134-136). Although no change in expression of RegIIIγ is detected following cefoperazone treatment of mice (52), other perturbations to the microbiota can have different effects on AMP levels (137) .

Beyond acute infections, the microbiota also influences adaptive immune responses to CDI. For example, IgA, specifically secretory IgA (sIgA) in the gut, is

important in mucosal immunity during gastrointestinal infections, as mice lacking sIgA have increased bacterial epithelial translocation (34). Interestingly, secretory IgA response is dependent on the resident microbiota and is adaptive to the changing microbiota composition (138, 139). Secretory IgA has been shown to contribute to protection against CDI, likely by providing additional protection to the epithelial barrier (140). However mice lacking the polymeric immunoglobulin receptor to transcytose antibodies across the epithelium reveal that though sIgA responses may contribute to protection, they are not required (140). Studies investigating the role of serum IgA and IgG against *C. difficile* toxins have debated the importance anti-toxin A versus anti-toxin B antibodies. For instance, in a case-control study of two geographically distinct cohorts, IgA levels against only toxin B were increased in cases versus controls (141). However, human monoclonal antibodies against both toxins A and B have been shown to significantly reduce CDI recurrence rate (142). Overall, the significance of the adaptive immune response in CDI is not completely understood, and more work is necessary to elucidate the role of the microbiota in shaping these responses against CDI.

Studying Colonization Resistance as a Community Effect

The processes that determine *C. difficile's* colonization are multilayered and involve a complex network of bacteria each with varying influence over *C. difficile's* fate. Total colonization resistance cannot be accurately measured in the context of one species' effect on another. Studies using multi-species probiotics against murine CDI show the synergistic protective effect of a bacterial consortium over its individual

members (117, 143). In studying the microbiota in health and disease, mathematical modeling is a useful tool to measure the importance of multiple factors in determining disease outcome. For example, a model built using data on a patient's microbiota structure accurately predicts psoriasis diagnosis (144). Mathematical modeling of a murine CDI model was also largely successful in predicting microbiota dynamics over time (145). These tools provide a potential means to predict the level of colonization resistance based on the microbiota structure. This would significantly improve CDI risk assessments and inform the design of multi-species probiotics.

Summary and Chapter Outline

The microbiota plays an integral role in colonization resistance against pathogen invasion. Alterations to the structure of the microbiota can result in host susceptibility to *C. difficile*. It is poorly understood what microbiota changes are necessary to occur for this loss in colonization resistance. The focus of this dissertation is to determine which members of the microbiota significantly contribute to colonization resistance and characterize *C. difficile*'s interaction with the microbiota.

In Chapter II, we characterize the microbiotas of human subjects with and without CDI. The microbiota of individuals with CDI had increased levels of *Enterococcus* and *Escherichia coli*, while control microbiotas contained higher levels of *Bacteroides*. Using information about the structure of the microbiota, we improve accuracy of models predicting *C. difficile* diagnosis based on patients' epidemiological data alone. This shows the impact of the microbiota as a whole in assessing *C. difficile* infection status.

In Chapter III we examine the effects of antibiotic perturbation on the murine gut microbiota structure and subsequent colonization resistance level against *C. difficile*. Several types and degrees of antibiotic manipulations were used to narrow in on bacteria responsible for the levels of *C. difficile* observed following challenge of these communities. *Escherichia*, similarly to what is observed in humans, has a positive relationship with *C. difficile*. Alternatively, Porphyromonadaceae in mice are the predominant Bacteroidales involved in resistance to colonization and growth. Using the microbiota structure data, we show the ability of a regression based model to accurately predict subsequent *C. difficile* colonization and growth levels 24 hours post challenge.

Understanding the interactions between *C. difficile* and the microbiota is crucial towards designing effective microbiota manipulation therapies. While FMTs are often successful in resolving CDI, a tailored multi-species probiotic approach would ensure transmission of an effective, safe, and compatible bacterial community. Given common themes among gut pathogen – microbiota – host interactions, these *C. difficile* studies may have broader applications for the microbiota in colonization resistance against other gastrointestinal pathogens.

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CHAPTER II

Microbiome data distinguishes patients with *Clostridium difficile* infection and non-*C. difficile* associated diarrhea from healthy controls

Abstract

Antibiotic usage is the most commonly cited risk factor for hospital-acquired *Clostridium difficile* infections (CDI). The increased risk is due to disruption of the indigenous microbiome and a subsequent decrease in colonization resistance by the perturbed bacterial community; however, the specific changes in the microbiome that lead to increased risk are poorly understood. We developed statistical models that incorporated microbiome data with clinical and demographic data to better understand why individuals develop CDI. The 16S rRNA genes were sequenced from the feces of 338 individuals, including cases, diarrheal controls, and non-diarrheal controls. We modeled CDI and diarrheal status using multiple clinical variables including age, antibiotic use, antacid use, and other known risk factors using logit regression. This base model was compared to models that incorporated microbiome data, using diversity metrics, community types, or specific bacterial populations, to identify characteristics of the microbiome associated with CDI susceptibility or resistance. The addition of microbiome data significantly improved our ability to distinguish CDI status when comparing cases or diarrheal controls to non-diarrheal controls. However, only when e

assigned samples to community types was it possible to differentiate cases from diarrheal controls. Several bacterial species within the Ruminococcaceae, Lachnospiraceae*, Bacteroides,* and Porphyromonadaceae were largely absent in cases and highly associated with non-diarrheal controls. The improved discriminatory ability of our microbiome-based models confirms that factors affecting the microbiome influence CDI.

Introduction

Since the discovery of penicillin in 1928, antibiotics have revolutionized health care, saving patients from life threatening infections such as bacteremia, bacterial meningitis, tuberculosis, and pneumonia. It has recently been estimated that over 250 million courses of antibiotics are prescribed to outpatients in the United States annually (1). However besides eradicating the pathogen of interest, antibiotics also disturb members of the indigenous bacterial community of the gastrointestinal tract, i.e. the gut microbiome. In hospitals, this disruption may result in *Clostridium difficile* infection (CDI), the leading nosocomial infectious disease in the United States. Cases of CDI have more than doubled since 2001 with over 300,000 new diagnoses in 2009 (2).

An intact microbiome is crucial for its role in providing colonization resistance against *C. difficile*. Antibiotic use, proton pump inhibitors, and advancing age, which are all known to influence the composition of the gut microbiome, are all risk factors for CDI (3-8). However, no one has developed a set of microbiome-based biomarkers for CDI to complement these risk factors. Therefore, characterizing the differences in the microbiome of individuals with and without CDI is essential towards understanding the

changes within the microbiome associated with CDI. This knowledge would also potentially lead to novel targeted therapies as the typical treatments of metronidazole and vancomycin feed the cycle of disrupting the gut microbiome. For the estimated 25% of cases of CDI-characterized recurrent infection, the most effective treatment has been fecal microbial transplant (FMT), which has a 92% success rate in limiting further recurrence (9). The remarkable success of FMT, which restores the normal microbiome, underscores the importance of understanding the role of the microbiome in providing colonization resistance.

Current microbiome-related studies use three general methods to characterize differences in the microbiome between groups of individuals. First, the microbial community composition of an individual can be distilled into a single parameter (i.e. alpha diversity) to describe the community in terms of richness or diversity. For instance, it has been shown that individuals with diarrhea tend to have a less diverse community composition than healthy individuals (10-11). Unfortunately, such results do not lend themselves to subsequent mechanistic investigations and do not provide a therapeutic avenue since it is difficult to predict whether an antibiotic will increase or decrease an individual's diversity. Second, cross-community comparisons have been made (i.e. beta diversity) to relate the similarity of microbial communities between individuals (e.g. UniFrac, Bray-Curtis). In humans, these metrics are useful in tracking an individual's recovery from antibiotic therapy (6); however, they have had limited use in discriminating between treatment groups (e.g. 12). Using beta diversity metrics, it is again difficult to develop a mechanistic understanding of the relationship between the community structure and disease or to provide a therapeutic avenue to change a

community structure. Finally, in an approach similar to genome wide association studies, comparisons of the relative abundance of individual bacterial populations can be made between groups of individuals (10,13). This approach does not account for the possibility that mixtures of populations can be protective or causative or that different mixtures can have the same phenotype in different individuals. Here we propose a comprehensive modeling approach that incorporates clinical metadata to identify collections of bacteria that can be associated with health and disease. A similar approach was recently used to model the microbiome to identify microbiome signatures of psoriasis; however, clinical data for the subjects was not included (14).

To better understand how clinical and microbiome-based factors are associated with CDI, we characterized the gut microbiome of hospitalized individuals who developed diarrhea with and without CDI and healthy individuals from the broader community. We used clinical and microbiome data to generate models of CDI status in order to differentiate between the three groups of subjects. Addition of microbiome data to clinical-based models for CDI significantly improved the ability to differentiate these patient groups. Using these models as tools, we identified bacteria with potential roles in colonization resistance against *C. difficile*, while controlling for clinically relevant risk factors.

Results

Patient sampling and base model framework

Fecal samples were collected from 338 individuals. Within this collection 183 diarrheal stool samples were acquired from inpatients at the University of Michigan

Hospital including both subjects with CDI (n=94) and without CDI (n=89). These samples were tested as positive or negative for *C. difficile* by the clinical microbiology lab at the hospital and subsequently confirmed through PCR using *C. difficile* specific 16S rRNA primers (See Materials and Methods). The remaining 155 non-diarrheal control stool samples were collected from individuals in the surrounding community. We collected a broad set of clinical data including risk factors for development of CDI from the subjects' questionnaire responses and their medical records (**Table 2.1**). As expected, antibiotic usage was more prevalent in CDI cases than individuals in either of the control groups ($p<0.001$). Fluoroquinolones represented the most used at-risk antibiotic in hospitalized patients, followed by amoxicillin and cephalosporins. Interestingly, individuals that were CDI positive were more likely to have lived with a healthcare worker. Together, these clinical data represented the framework for our base model.

We used age, gender, race, antibiotics, antacids, vegetarian diet, surgery within the past 6 months, a history of CDI, residence with another person who had CDI, and residence with another person who works in the health care setting as explanatory variables for three logit models. For each model, we evaluated their ability to discriminate group classification using the area under the receiver operator characteristic (ROC) curve (AUC) (15). These curves look at the true positive rate, i.e. sensitivity, as it relates to the false positive rate, i.e. 1-specificity. Using the full collection of explanatory variables we were interested in three comparisons. The first comparison differentiated between the cases and the non-diarrheal controls (AUC=0.891). The second differentiated between the cases and diarrheal controls

Characteristic	Case (n=94)	Diarrheal Control (n=89)	Non-Diarrheal Control (n=155)	*P*-value
Sex				
Females, n (%)	53 (56.4%)	49 (55.1%)	102 (65.8%)	
Males, n (%)	41 (43.6%)	40 (44.9%)	53 (34.2%)	0.166
Age				
Mean years (SD)	55.9 (18.3)	58.7 (14.9)	52.2 (21.5)	0.034
Range years	18 - 89	18 - 85	19 - 88	
Race, n (%)				0.712
White	84 (89.4%)	76 (85.4%)	129 (83.2%)	
Black	7 (7.4%)	9 (10.1%)	16 (10.3%)	
Other/Unknown	3 (3.2%)	4 (4.5%)	10 (6.5%)	
Weight, mean lbs (SD)	169.9 (56.9)	177.9 (54.5)	171.5 (47.3)	0.549
Vegetarian, n (%)	2 (2.1%)	5 (5.6%)	8 (5.2%)	0.435
Antibiotics <3 months, n (%)	72 (76.6%)	56 (62.9%)	21 (13.5%)	<0.001
Fluoroquinolone, n (%)	21 (22.3%)	17 (19.1%)	4 (2.6%)	<0.001
Amoxicillin, n (%)	10 (10.6%)	6 (6.7%)	7 (4.5%)	0.182
Cephalosporin, n (%)	11 (11.7%)	3 (3.4%)	3 (1.9%)	0.004
Clindamycin, n (%)	2 (2.1%)	0	5 (3.2%)	0.297
Ampicillin, n (%)	1 (1.1%)	0	0	0.541
Antacids <30 days, n (%)	20 (21.3%)	20 (22.5%)	11 (7.1%)	0.001
Surgery <6 months, n (%)	48 (51.1%)	38 (42.7%)	14 (9.0%)	<0.001
History of *C. difficile*, n (%)	1 (1.1%)	2 (2.2%)	4 (2.6%)	0.793
Reside with person with CDI, n (%)	1 (1.1%)	2 (2.2%)	1 (0.6%)	0.593
Reside with healthcare worker, n (%)	25 (26.6%)	13 (14.6%)	17 (11.0%)	0.005

TABLE 2.1 Demographic information for subjects in each experimental group

(AUC=0.659). The third differentiated between the diarrheal and non-diarrheal controls (AUC=0.849). The base model for cases and diarrheal controls was the only comparison that was not significantly different than an empty model (i.e. a model without independent variables; p=0.189). The AUC and 95% confidence intervals for all models are listed in **Table 2.2**. These three models served as the base for our development of other logit models that incorporated microbiome-based data.

Incorporation of diversity measures into logit models

We first looked at the overall microbiome structural differences among the individuals in our study (**Figure 2.1**). There were apparent structural differences between non-diarrheal control samples and hospital-acquired samples (cases and diarrheal controls). These differences were statistically significant by analysis of molecular variance (AMOVA) ($p<0.001$). The structure of cases and diarrheal controls were additionally significantly different from one another by AMOVA ($p=0.02$), although to a lesser degree. In order to identify the differences between these experimental groups, we first looked at their levels of overall bacterial diversity. Previous studies have shown that the bacterial diversity of subjects with initial and recurrent CDI is markedly lower than that of healthy subjects (10-11). Measuring diversity using the inverse Simpson index, we found that hospital-acquired samples had a 2-fold lower diversity compared to the non-diarrheal controls, but were not significantly different from each other (**Figure 2.2A**). A model based on the inverse Simpson index alone significantly differentiated non-diarrheal controls from either cases or diarrheal controls (**Figure 2.2B-D**); although this model performed no better than the base model alone.

Contrasts:	AUC	95% CI	*P*-value
Base Model			
Cases vs. Diarrheal Controls	0.659	0.580, 0.738	0.189*
Cases vs. Non-diarrheal Controls	0.891	0.849, 0.933	**<0.0001***
Diarrheal Controls vs Non-diarrheal	0.849	0.795, 0.903	**<0.0001***
Inverse Simpson Alone			
Cases vs. Diarrheal Controls	0.580	0.495, 0.665	0.554*
Cases vs. Non-diarrheal Controls	0.809	0.754, 0.865	**<0.0001***
Diarrheal Controls vs Non-diarrheal	0.811	0.751, 0.871	**<0.0001***
Base + Inverse Simpson			
Cases vs. Diarrheal Controls	0.665	0.586, 0.744	0.577**
Cases vs. Non-diarrheal Controls	0.922	0.889, 0.955	**0.0072****
Diarrheal Controls vs Non-diarrheal	0.900	0.858, 0.942	**0.0009****
Community Types Alone			
Cases vs. Diarrheal Controls	0.698	0.623, 0.773	**0.0144***
Cases vs. Non-diarrheal Controls	0.896	0.855, 0.938	**<0.0001***
Diarrheal Controls vs Non-diarrheal	0.828	0.774, 0.882	**<0.0001***
Base + Community Types			
Cases vs. Diarrheal Controls	0.763	0.694, 0.831	**0.0059****
Cases vs. Non-diarrheal Controls	0.957	0.934, 0.981	**0.0002****
Diarrheal Controls vs Non-diarrheal	0.905	0.852, 0.958	**0.0035****
Specific OTUs Alone			
Cases vs. Diarrheal Controls	0.696	0.619, 0.773	0.0934*
Cases vs. Non-diarrheal Controls	0.950	0.922, 0.978	**<0.0001***
Diarrheal Controls vs Non-diarrheal	0.981	0.967, 0.995	**<0.0001***
Base + Specific OTUs			
Cases vs. Diarrheal Controls	0.709	0.634, 0.784	0.0652**
Cases vs. Non-diarrheal Controls	0.985	0.973, 0.996	**<0.0001****
Diarrheal Controls vs Non-diarrheal	0.983	0.971, 0.996	**<0.0001****

Base model included age, gender, race, antibiotics, antacids, surgery in last 6 months, history of *C. difficile,* vegetarian, residence with person with *C. difficile*, residence with healthcare worker.

* *P*-value for the difference in areas under ROC curves between an empty model versus the model listed.

** *P*-value for the difference in areas under ROC curves for the Base Model versus the Base Model with the microbiome data listed.

TABLE 2.2 AUC comparisons of all models

The AUC values and 95% confidence intervals were calculated for each model in our study. Comparisons were made either between the listed model and an empty model (*) or between a combined base plus microbiome model and the base model (**), as indicated above. Comparisons that were significant are shown as bold p-values.

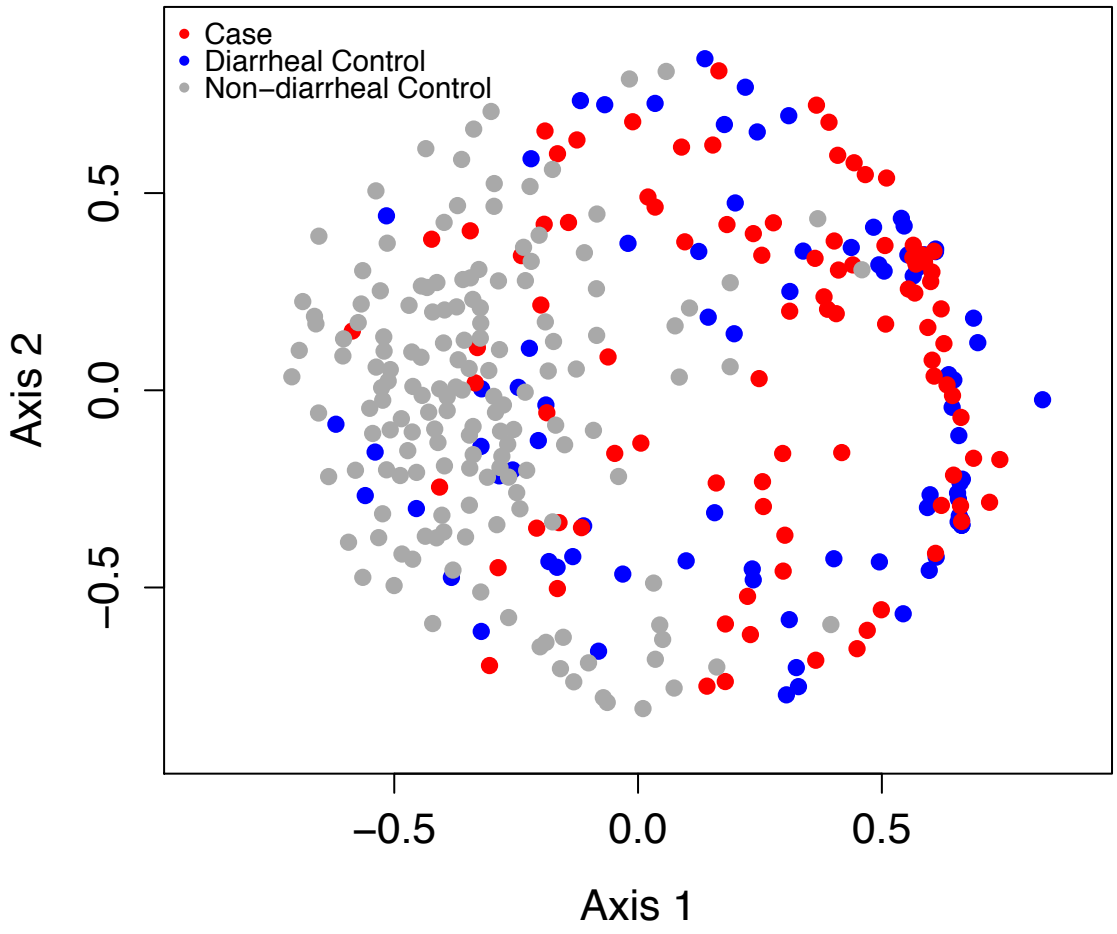

FIGURE 2.1 Overall structural differences between experimental groups
This non-metric multidimensional scaling (NMDS) plot was generated from the distance matrix of θ_{YC} values that assess structural differences between samples (40). Gray represents non-diarrheal control samples, blue are diarrheal control samples, and red are case samples.

When we incorporated the inverse Simpson index into our base models, differentiation of cases from non-diarrheal controls was significantly improved (AUC=0.922, p=0.0072), and differentiation of diarrheal controls from non-diarrheal controls was also significantly improved (AUC=0.900, p=0.0009). Cases and diarrheal controls were indistinguishable when we used models that incorporated the inverse Simpson index. We performed the same analysis using the Shannon diversity index and observed similar results. These results indicate that although low diversity was a characteristic of CDI positive subjects, subjects with diarrhea had lower diversity than healthy outpatients.

Incorporation of bacterial community types into logit models

Next, we sought to determine whether a subject's overall community composition differentiated CDI or diarrheal status. We assigned the samples to a specified number of clusters (k=2-15) based on their similarity to other samples after removing the *C. difficile* OTU (OTU 19). We selected 13 as the appropriate number of clusters (i.e. community types) as this resulted in the optimal AUC for the base model when we incorporated the subject's community type. These community types varied in the prevalence of CDI among individuals within each type (See **Figure 2.3A**, percent of case subjects). Results from models using community types alone performed similarly to that of the base models (**Figure 2.3B-D**). When the community type assignments were added to the base models, the AUCs significantly improved relative to the base models in all comparisons (**Table 2.2**). These results indicate that specific community types differentiated CDI status and suggest that certain community types may be

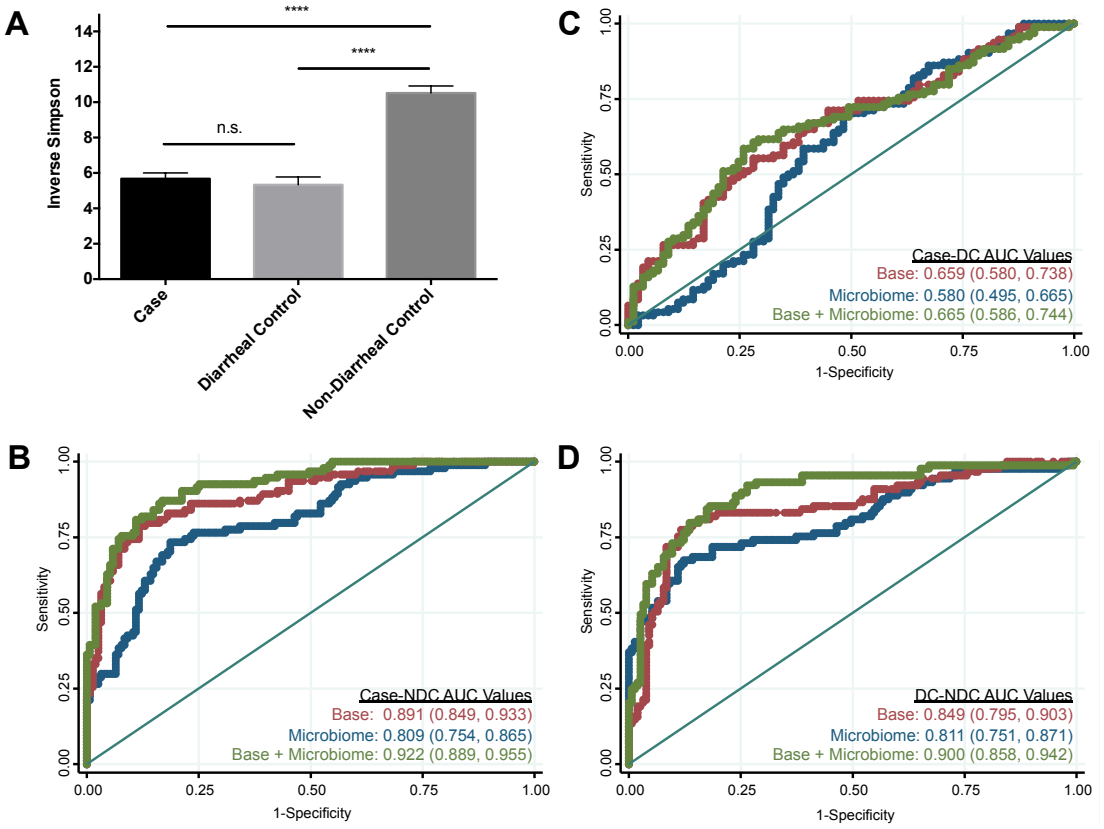

FIGURE 2.2 Bacterial diversity distinguishes between subjects with and without diarrhea

A. Alpha diversity was measured using the inverse Simpson index. Statistical analysis was performed using Dunn's multiple comparison test. **** Indicates $p < 0.0001$, n.s. indicates no significance, error bars represent +/- SEM. **B-D.** ROC curves and AUC values with 95% confidence intervals in parenthesis for each model comparing (**B**) case versus non-diarrheal control (NDC), (**C**) case versus diarrheal control (DC), and (**D**) diarrheal control versus non-diarrheal control. Red represents the base model, blue represents the inverse Simpson model, and green represents the base + inverse Simpson model. The straight line represents the null model.

more susceptible to colonization by *C. difficile*.

To determine the taxonomic composition of each of these community types, we used the random forest feature selection algorithm to identify those taxa that were indicators for the different community types (**Figure 2.3A**). There were 6 community types that were less prevalent among case individuals or diarrheal controls relative to the non-diarrheal controls (i.e. Types 12, 3, 9, 7, 13, and 11). These 6 types had a higher relative abundance of OTUs belonging to the *Bacteroides* genus (OTUs 3, 4, 5, 8), *Alistipes* (OTU 6), *Prevotella* (OTU 17), and Ruminococcaceae (OTU 7) than the other community types. While the remaining 7 types (i.e. 1, 8, 10, 4, 6, 5, 2) were enriched in Enterobacteriaceae (OTU 1), *Enterococcus* (OTU 2), *Blautia* (OTU 11), and Lachnospiraceae (OTU 13). There were 6 community types that were less prevalent among case individuals relative to the diarrheal controls (i.e. Types 6, 5, 2, 9, 7, and 11). These types could be further subdivided by the overall percentage of diarrheal controls within each type. Types 6, 5, and 2 had a high percentage of diarrheal controls, while types 9, 7, and 11 had a low percentage of diarrheal controls (**Table 2.3**). Types 6, 5, and 2 were also low in non-diarrheal controls and lacked several OTUs found primarily in that group (**Figure 2.3A**). Furthermore, types 2 and 6 were highly enriched in either *Enterococcus* or Enterobacteriaceae, respectively. Types 9, 7, and 11 also had a high proportion of non-diarrheal controls and were more abundant in OTUs found predominantly in the non-diarrheal control group. These group-specific taxonomic features we have identified may be involved in susceptibility or resistance to CDI.

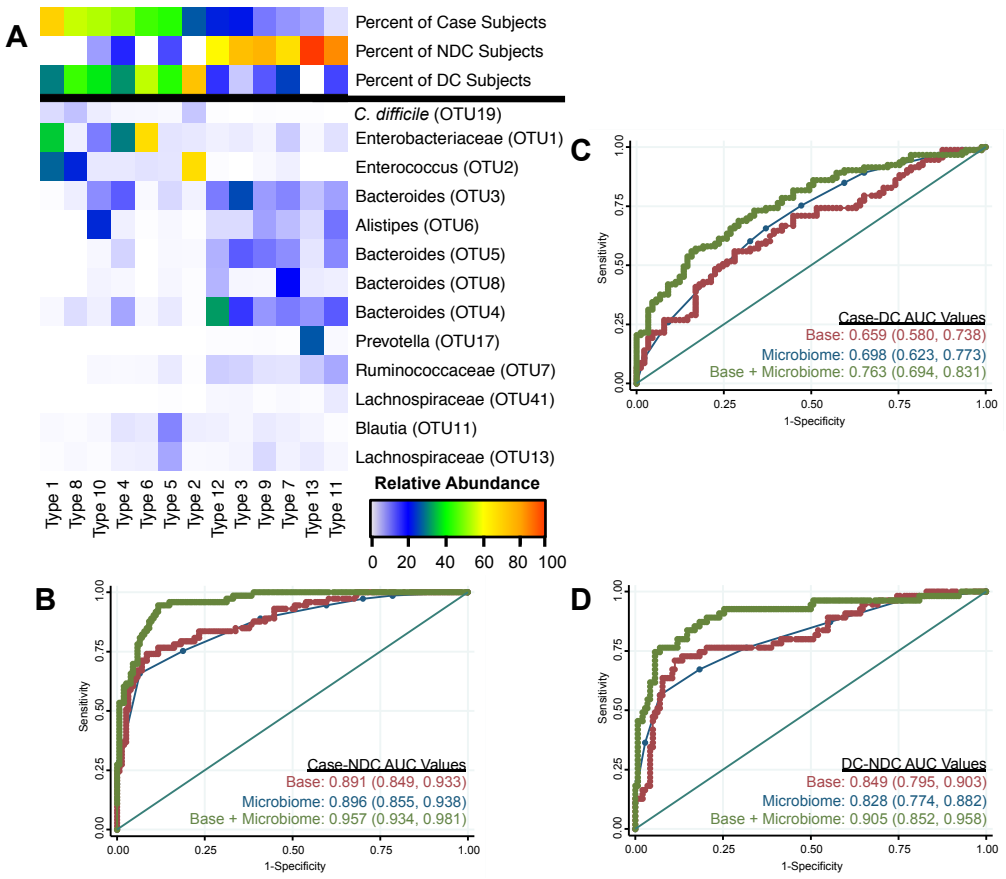

FIGURE 2.3 Specific community structure types significantly differentiate all population comparisons

A. Heat map showing the structural differences between each community type. The top 3 rows show the percentage of individuals classified as a case, diarrheal control, or non-diarrheal control across each type. The remaining rows show the relative abundance for OTUs identified using feature selection through random forest analysis. Although the relative abundance of *C. difficile* OTU 19 is shown, it was not considered in the formation of these community types. Types are ordered by decreasing percentage of case individuals. **B-D.** ROC curves and AUC values with 95% confidence intervals in parenthesis for each model comparing (**B**) case versus non-diarrheal control, (**C**) case versus diarrheal control, and (**D**) diarrheal control versus non-diarrheal control. Red represents the base model, blue represents the community types model, and green represents the base + community types model. The straight line represents the null model.

Community Type	#Diarrheal Controls	#Non-Diarrheal Controls	#Cases	Total n	%Diarrheal Control	%Non-Diarrheal Control	%Case
1	6	0	14	20	30.0	0.0	70.0
8	4	0	5	9	44.4	0.0	55.6
10	5	1	7	13	38.5	7.7	53.8
4	11	6	18	35	31.4	17.1	51.4
6	11	0	9	20	55.0	0.0	45.0
5	9	3	9	21	42.9	14.3	42.9
2	19	0	7	26	73.1	0.0	26.9
12	5	19	7	31	16.1	61.3	22.6
3	2	34	10	46	4.3	73.9	21.7
9	5	29	4	38	13.2	76.3	10.5
7	6	16	2	24	25.0	66.7	8.3
13	0	13	1	14	0.0	92.9	7.1
11	6	34	1	41	14.6	82.9	2.4

TABLE 2.3 Breakdown of the individuals in each community type
All 338 subjects in the study were sorted into 13 community types using PAM (see Materials and Methods). These 13 community types were ordered by the percentage of case individuals belonging to each type. The number of diarrheal control, non-diarrheal control, and case subjects belonging to each community type are shown.

Incorporation of specific bacterial populations into logit models

Having established that incorporation of a subject's community type could better inform their CDI or diarrheal status than a diversity index, we attempted to determine whether more specific components of those community types could improve our models. To accomplish this, we first identified those bacterial populations that were differentially represented in each of the three comparisons using LEfSe (Linear Discriminant Analysis Effect Size) (13); these analyses excluded *C. difficile* (OTU 19). Briefly, LEfSe uses the Kruskal-Wallis sum-rank test to identify taxonomic features that characterize the differences between our study groups and linear discriminant analysis to evaluate the effect size of each feature. Within each comparison, the OTUs with the largest effect size in each group and comparison were included in the respective base model (see **Figure 2.4A**).

Within the case versus non-diarrheal control comparison, 13 OTUs were significantly enriched in cases and 26 OTUs were significantly enriched in the non-diarrheal controls (**Figure 2.4A**). We selected 5 OTUs that were enriched in the cases and 5 that were enriched in the non-diarrheal controls (based on the most extreme differences) and included their relative abundance as independent variables in logit regression. The relative abundance of these specific OTUs discriminated quite well between cases and non-diarrheal controls (AUC=0.950, **Table 2.2**). Additionally the combined base-microbiome model significantly outperformed the base model (AUC=0.985, $p<0.0001$). Subjects having CDI were significantly more likely to harbor *Enterococcus* (OTU 2), Lachnospiraceae (OTU 14), Erysipelotrichaceae (OTU 22), and significantly less likely to harbor *Bacteroides* (OTU 5) than non-diarrheal controls.

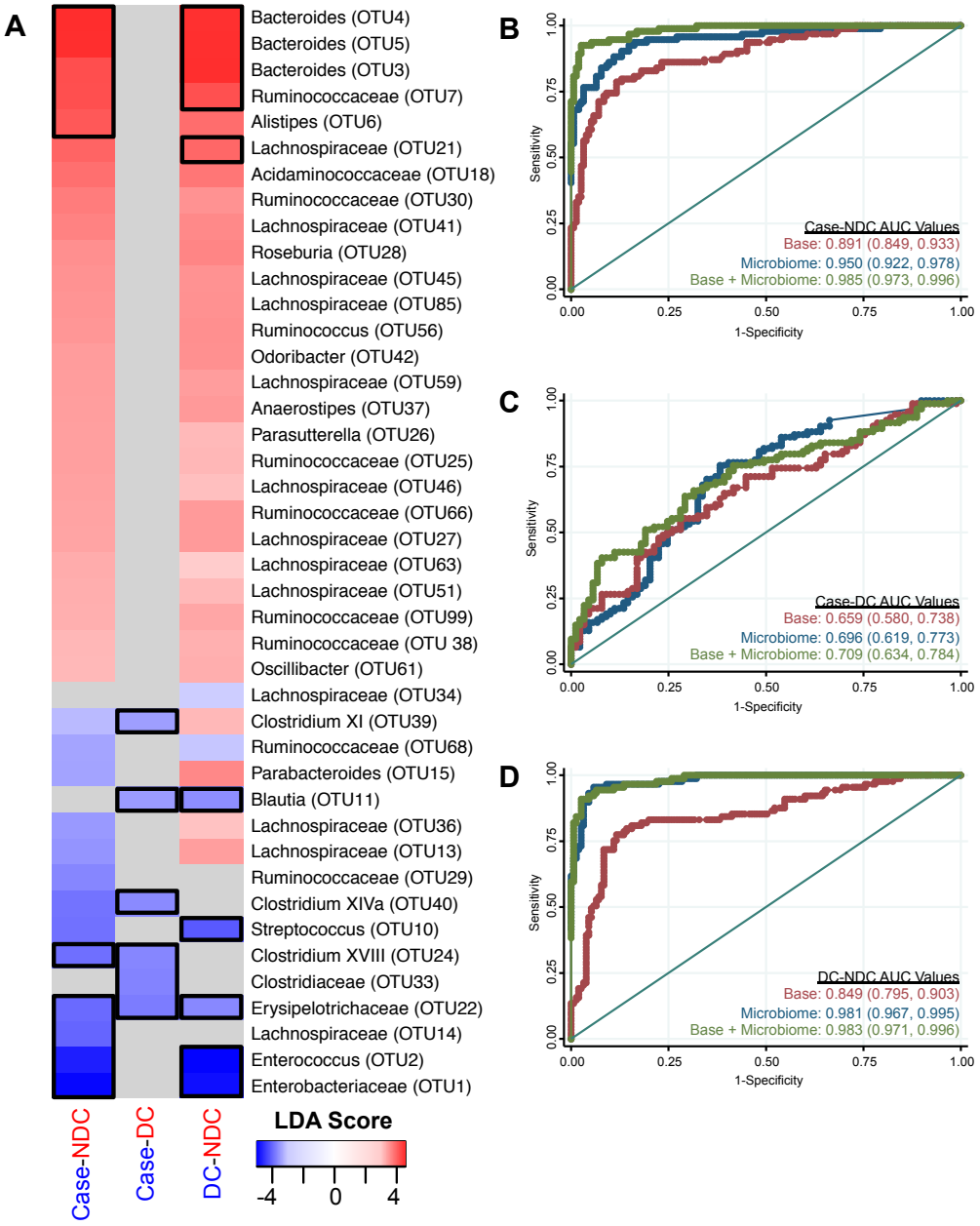

FIGURE 2.4 Specific bacterial populations clearly differentiate non-diarrheal controls from subjects with CDI and non-CDI associated diarrhea

A. LEfSe was used to compare cases and non-diarrheal controls, cases and diarrheal controls, and diarrheal controls and non-diarrheal controls. Only the LDA scores of significant OTUs are shown. Grey boxes indicate not significant for given comparison. Number following bacterial name indicates OTU number. Black boxes show the OTUs at the 0.25%-iles cutoff for each group that were chosen for inclusion in the microbiome models. DC, diarrheal control; NDC, non-diarrheal control. **B-D.** ROC curves and AUC values with 95% confidence intervals in parenthesis for each model comparing (**B**) case versus non-diarrheal control, (**C**) case versus diarrheal control, and (**D**) diarrheal

control versus non-diarrheal control. Red represents the base model, blue represents the specific OTUs model, and green represents the base + specific OTUs model. The straight line represents the null model.

These results confirm the differences that were observed between the case and non-diarrheal control enriched community types and highlight the populations that had the greatest contribution to the model.

In the comparison of cases and diarrheal controls, no OTUs were significantly enriched in the diarrheal controls over the cases; however, we identified 6 OTUs that were significantly enriched in the cases (**Figure 2.4A**). The relative abundances of these OTUs, when combined in a logit model (**Figure 2.4C**), did not significantly distinguish cases and diarrheal controls (AUC=0.696, p=0.0934). Furthermore, the base plus microbiome model was not significantly different from the base clinical model (AUC=0.709, p=0.0652). Unlike the bacterial community type analysis, we were unable to identify specific structural differences that could distinguish between cases and diarrheal controls in this model. These results confirm that overall microbiome structure was more discriminatory for patients with non-*C. difficile* associated and *C. difficile* associated diarrhea.

Finally, in the comparison of diarrheal controls and non-diarrheal controls we identified 30 OTUs that were enriched in the non-diarrheal controls and 7 OTUs that were enriched in the diarrheal controls (**Figure 2.4A**). Individuals with non-*C. difficile* associated diarrhea were more likely to have higher relative abundances of Enterobacteriaceae (OTU 1), *Enterococcus* (OTU 2), Erysipelotrichaceae (OTU 22), *Streptococcus* (OTU 10), and *Blautia* (OTU 11). The non-diarrheal controls were more likely to have higher levels of several *Bacteroides*, Lachnospiraceae, and Ruminococcaceae OTUs. These taxa are commonly associated with a healthy microbiome. We used the 5 most enriched OTUs in each of the diarrheal control or the

non-diarrheal control group to create a logit model to differentiate between the two (**Figure 2.4D**). These OTUs significantly differentiated the two control groups (AUC=0.981). The inclusion of both clinical data and these OTUs provided considerable discrimination between the two groups as compared with the base model alone (AUC=0.983; $p<0.0001$). These results indicate that there were significant changes to the microbiome when individuals had diarrhea.

Discussion

We found distinct differences in the microbiome of people with and without CDI as well as with and without diarrhea. We developed classification models to differentiate whether individuals had *C. difficile* infection or non-*C. difficile* associated diarrhea based on clinical and microbiome data. The microbiome was incorporated into these models using three approaches: diversity indices, community types, and defined bacterial subsets. These approaches of representing the microbiome allowed us to describe the communities at varying levels of resolution. When differentiating between the cases and diarrheal controls, incorporation of community types provided the only significant improvement in detection between these two groups of patients. The inverse Simpson index and the utilization of specific OTUs did not differentiate these two groups. For the comparisons of non-diarrheal controls to cases or diarrheal controls, inclusion of the microbiome data significantly enhanced our ability to differentiate the groups regardless of the approach we used to represent the microbiome. The highest AUCs were observed when differentiating between hospitalized patients (either cases or diarrheal controls) and community residents (non-diarrheal controls), with AUCs

consistently greater than 0.9 when both clinical and microbiome data were considered. Specifically, representing the microbiome using specific sets of OTUs was the best approach for differentiating between hospitalized patients and community subjects. These findings stress the importance of not just one individual bacterial population or one metric of the community (e.g. diversity), but rather collections of bacterial populations or overall community types in detecting disease state. They also suggest the presence of gut dysbiosis in patients with diarrhea. These results demonstrate that knowledge of bacterial communities, not just single species, and in combination with clinical factors may be beneficial in generating epidemiological models of disease.

Of our three comparisons, it was notable that the cases and diarrheal controls were the most similar. First, the base model to differentiate the two groups was unable to perform significantly better than a null model ($p=0.19$). Second, the inverse Simpson diversity index revealed similar levels in both groups ($p=0.85$). Third, many community types characterized by high numbers of cases were also more likely to contain diarrheal controls than non-diarrheal controls, which tended to cluster separately from both diarrheal groups. Finally, our OTU-based analysis did not identify any OTUs as being significantly enriched in the diarrheal controls relative to the cases. Because of the similarity in community structure and in clinical risk factors for CDI, results suggest that many of the diarrheal control subjects may actually be susceptible to CDI and have not yet been exposed to *C. difficile*. This implies that any perturbation resulting in diarrhea may also contribute to CDI. This hypothesis is particularly relevant within a hospital setting where *C. difficile* spores are abundant and where there are numerous potential

causes of diarrhea including antibiotics (independent of CDI), infection, chemotherapy, and dietary changes.

Our models that compared cases to non-diarrheal controls showed that *Bacteroides*, Lachnospiraceae, and Ruminococcaceae were enriched in controls and *Enterococcus*, Enterobacteriaceae, Erysipelotrichaceae, and some Lachnospiraceae were enriched in cases. These results confirm previous related studies (10, 16-18). Members of the Lachnospiraceae and Ruminococcaceae are the primary butyrate-producing bacteria in the human gastrointestinal tract. Butyrate has been associated with inhibition of *C. difficile* growth in vitro (19), inflammation suppression, and the health of colonic cells. Thus, butyrate as well as other short-chain fatty acids may represent one mechanism of colonization resistance. Comparison of the sequences within our *Bacteroides* OTU (OTU 5), which was enriched in our non-diarrheal controls, to an annotated 16S rRNA gene database showed that they were highly similar to *B. uniformis* and *B. acidifaciens*. *B. uniformis* was previously shown to ameliorate metabolic dysfunction caused by diet-induced obesity via changes in metabolic and immune responses (20). Because obesity is a risk factor for CDI (21), it is possible that *B. uniformis* would also provide protection against infection by *C. difficile*. *B. acidifaciens* was demonstrated to increase IgA+ B cells in the large intestine (22), which may also limit the growth of gastrointestinal pathogens such as *C. difficile*. Overall, this shift in community structure is thought to be associated with a change in colonization resistance. Murine models of CDI have observed that similar changes in community structure render normally resistant mice sensitive to colonization by *C. difficile* (23-24). Similarly, a mixture of 6 bacterial species that included a member of the *Bacteroides*

genus and a member of the Lachnospiraceae, which were both found significantly enriched in our non-diarrheal control population, was sufficient to clear *C. difficile* in a murine model of recurrent CDI (17).

Microbiome analyses have revealed that bacterial populations are patchy across individuals and that there is no "core microbiome" (12). This hinders one's ability to consistently associate specific bacterial populations with disease. Instead, others have developed the concept of community or enterotypes (25-27). Although the biological interpretation of these clusters is controversial, our study demonstrates that categorizing individuals into community types or utilizing subsets of the bacterial community improves our ability to identify individuals that belong to specific disease states. Similar approaches have been used to associate specific community types with the composition of one's diet, obesity, inflammatory bowel disease, Crohn's disease, *Trichomonas vaginalis* infection, vaginal pH, and ethnicity (25, 27-32); however, these studies have not combined the subject's clinical information and community type to evaluate disease state.

The models evaluated in this study reflect bacterial communities at a specific point in time for these three patient groups. Thus we are limited in our ability to assess the contribution of the microbiome towards risk or prevention of CDI. We also cannot determine the length of time that cases were colonized by *C. difficile* prior to sample collection. The aim of this investigation was not to enhance CDI diagnostics, but to use a model-based framework to characterize features of the microbiome that are associated with CDI and health. Our approach suggests that knowledge of an individual's microbiome composition is useful in distinguishing disease from health.

However, prospective studies are needed to validate microbiome-based biomarkers of CDI risk. Identification of such risk factors will only be possible if samples are collected before the development of CDI. Furthermore, previous modeling has shown that albumin levels, white blood cell count, creatinine levels, age, and increased leukocyte count can be used to predict CDI severity and mortality (4, 9, 33). It is possible that the incorporation of microbiome data could also be used to improve predictions of disease outcome. However in the current investigation we did not collect sufficient CDI severity data to address this question. As we have demonstrated in this study, there are distinct microbiome signatures that are associated with CDI. Understanding which community-wide changes are responsible for the loss in colonization resistance leading to CDI is critical for future risk models and therapeutics.

Materials and Methods

Sample collection and definitions.

This study was approved by the University of Michigan Institutional Review Board. The inpatient samples were collected from October 2010 to January 2012 at the University of Michigan Hospital, Ann Arbor, Michigan. All enrollees granted patient consent. Inpatient subjects were not pregnant, were suspected of having an initial episode of CDI (not recurrent CDI), and their stool sample was diarrheal. Within 24 hours of stool collection, these specimens were screened for *C. difficile* using the CDIFF QUIK CHEK COMPLETE® assay (TECHLAB, Blacksburg, VA). This rapid membrane enzyme immunoassay tests for the presence of both the *C. difficile* antigen glutamate dehydrogenase (GDH) as well as the *C. difficile* toxin proteins A and B. If this test

resulted in positive or negative result for both GDH and toxin proteins, the sample was classified as a case or as a diarrheal control, respectively. If the test was positive only for GDH, a PCR screen for the *C. difficile tcdB* gene, which encodes the toxin B protein, was performed (34). To confirm the results of the clinical lab, we additionally performed PCR on all inpatient samples using *C. difficile* specific 16S rRNA gene primers as described elsewhere (35). Non-diarrheal, *C. difficile* negative samples were collected between January 2011 and January 2012 from individuals residing in the surrounding Ann Arbor area. Subjects were excluded if they had any signs of diarrhea in the previous 7 days or were pregnant. Once enrolled, individuals collected a stool sample using the provided home stool specimen kit.

DNA sequencing and curation

Total bacterial DNA was extracted from each stool sample using the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) on an EpMotion 5075 liquid handling workstation (Eppendorf, Hamburg, Germany). The V35 region of the 16S rRNA gene was amplified and sequenced using 454 GS FLX pyrosequencing platform and curated using mothur as previously described (36-37). We sequenced and processed a mock community in parallel to the samples sequenced for this study (37). The observed error rate among the mock community samples was 0.009%. Sequences were clustered into operational taxonomic units (OTUs) using a 3% distance cutoff (38). Taxonomic assignments were determined by using a naïve Bayesian classifier with the RDP training set with an 80% bootstrap confidence threshold. To mitigate against the effects of uneven sampling, all samples were rarefied to 1450 sequences per sample

(37). Among the samples with more than 1450 sequences, the number of sequences per sample varied from 1450 to 17120 with a mean of 6091 sequences/sample, a median of 5986, and a median absolute deviation of 1296. The OTU corresponding to *C. difficile* (OTU19) was identified by checking the representative sequence against the nt NCBI database with blastn. All 16S rRNA gene sequence data and the associated MIMARKS table are available at http://www.mothur.org/CDI_MicrobiomeModeling.

Statistical analyses

Initial statistical analyses were conducted to assess differences among the three study groups (*C. difficile* cases, diarrheal controls and non-diarrheal controls). For continuous variables (e.g., age, weight) one-way analysis of variance was utilized. For categorical variables, Pearson's chi-squared test was calculated or Fisher's exact test when expected cell frequencies were less than or equal to 5. The principal intent of the analyses was to assess whether the addition of microbiome data added to case differentiation, and as such nested logit models were constructed with clinical data, with and without the incorporation of microbiome data. We utilized three approaches to capture the biodiversity of the gut microbiome. First, the inverse Simpson index was calculated for each sample and treated as a continuous variable in the models (39). Second, we assigned each sample to a different community type and used the type as categorical variables in the model. These community types were identified by partitioning around medoids (PAM) of a Jensen-Shannon divergence distance matrix calculated from the microbiome data (27). The randomForest package in R (http://cran.r-project.org/) with number of trees set to 1000 was used to differentiate the

composition of each cluster. Third, we built models using the relative abundances of a subset of the OTUs observed across the individuals. These OTUs were selected using LEfSe based on the comparisons of cases versus diarrheal controls, cases versus non-diarrheal controls, and diarrheal controls versus non-diarrheal controls (13). OTUs demonstrating the greatest differences (at a 0.25 percentile cutoff at both ends) were used as continuous variables in our logit models. The 0.25 percentile cutoff was selected to restrict the number of significant OTUs in order to build the models and avoid over-fitting. Differences between nested models were compared using the test for the equality of ROC areas (15). Analyses were conducted in Stata/MP 12.1 and R version 3.0.1.

Notes

This work was reprinted and modified with permission from Schubert A.M., Rogers M.A.M., Ring C., Mogle J., Petrosino J.P., Young V.B., Aronoff D.M., Schloss P.D. 2014. Microbiome data distinguishes patients with *Clostridium difficile* infection and non-*C. difficile* associated diarrhea from healthy controls. *mBio*. 5(3):e01021-14.

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CHAPTER III

Antibiotic-induced alterations of the murine gut microbiota and subsequent effects on colonization resistance against *Clostridium difficile*

Abstract

Perturbations to the gut microbiota result in a loss of colonization resistance against gastrointestinal pathogens such as *Clostridium difficile*. Although *C. difficile* infection is commonly associated with antibiotic use, the precise alterations to the microbiota associated with this loss in function are unknown. We used a variety of antibiotic perturbations to generate a diverse array of gut microbiota structures, which were then challenged with *C. difficile* spores. Across these treatments we observed that *C. difficile* resistance was never attributable to a single organism, but rather it was the result of multiple microbiota members interacting in a context-dependent manner. Using relative abundance data, we built a machine learning regression model to predict the levels of *C. difficile* that were found 24 hours after challenging the perturbed communities. This model was able to explain 77.2% of the variation in the observed number of *C. difficile* per gram of feces. This model revealed important bacterial populations within the microbiota, which correlation analysis alone did not detect. Specifically, we observed that populations associated with the Porphyromonadaceae, Lachnospiraceae, *Lactobacillus*, and *Alistipes* were protective and populations

associated with *Escherichia* and *Streptococcus* were associated with high levels of colonization. In addition, a population affiliated with *Akkermansia* indicated a strong context dependency on other members of the microbiota. Together, these results indicate that individual bacterial populations do not drive colonization resistance to *C. difficile*. Rather, multiple diverse assemblages act in concert to mediate colonization resistance.

Introduction

The microbiota, or the diverse community of microorganisms living in and on the body, has an integral role in deterring pathogen colonization and infection (1). This native protection by the microbiota from invasive pathogenic species is termed colonization resistance. It is well established that the gut bacterial microbiota is critical in the host's defense against the pathogen *Clostridium difficile* (2-4). Perturbations to this indigenous community often lead to a loss of resistance. This is especially important in many hospital settings where patients are not only exposed to various types and degrees of perturbations, such as antibiotics, diet changes, and chemotherapy, but they are also exposed to *C. difficile* spores from their environment (5). *C. difficile* infections (CDI) are the most reported hospital-acquired infection in the United States and are responsible for 14,000 deaths a year (6).

It is not completely understood how different perturbations to the gut microbiota result in a loss of colonization resistance to *C. difficile*. There is a clear need to better understand the ecology of *C. difficile* and its interactions with members of the microbiota. In mouse models of CDI, the unperturbed, untreated murine microbiome is

completely resistant to *C. difficile* colonization. It was previously shown that C57Bl/6 mice treated with cefoperazone (3, 7), tigecycline (8), clindamycin (9), or clindamycin in combination with a five antibiotic cocktail (2) had decreased colonization resistance. These studies suggest that a loss of Lachnospiraceae and *Barnesiella* and a bloom of Lactobacillaceae and Enterobacteriaceae are responsible for the loss of colonization resistance. These results are largely supported by human association studies (10, 11). We previously observed significant differences between the gut microbiota of hospitalized individuals with and without *C. difficile* and non-hospitalized controls (10). In addition, fecal microbiota transplants increase Bacteroidetes and decrease Proteobacteria levels in recipients, resulting in a successful restoration of colonization resistance in patients (12). Precisely how this occurs is not fully understood, but it emphasizes the importance of the gut microbiota in colonization resistance against *C. difficile*.

Because the gut microbiota is a complex community we need tools that enable us to dissect the interactions within the community and with *C. difficile*. One approach is the use of mathematical models to identify associations between members of the microbiota and *C. difficile*. Mathematical models have been used to predict *C. difficile* (10, 13) and *Citrobacter* infection (14), colon cancer (15), and psoriasis (16) based on the composition of the gut microbiota. We similarly sought to identify the subset of the normal murine microbiota that are responsible for colonization resistance by using mathematical models to explain the relationship between members of the gut microbiota.

The purpose of this investigation was to expand our current knowledge of the effects of various perturbations on colonization resistance against *C. difficile*. Through the administration of different antibiotic classes, doses, and recovery times we altered the murine gut microbiota and challenged the communities with *C. difficile* spores to quantify differences in colonization resistance. We then used 16S rRNA gene sequencing to identify structural changes within the microbiota that would be predictive of colonization resistance. Using these data, we built a random forest regression model to predict *C. difficile* colonization levels. Through this analysis, we have identified groups of related bacteria that are associated with *C. difficile* colonization resistance. This model revealed that the interactions giving rise to colonization resistance were non-linear and context dependent. These findings show we can successfully apply modeling techniques to accurately predict the colonization resistance of a given microbiota.

Results

Antibiotics differentially alter the structure of the microbiota and their colonization resistance to C. difficile

We selected a panel of seven antibiotics from six classes with the goal of differentially altering the microbiota and assessing their resistance to *C. difficile* colonization (**Table 3.1**). Following the cessation of antibiotics, each treatment group was given one day of recovery prior to challenge with *C. difficile* spores. One day post challenge we enumerated the density of *C. difficile* in the animals' feces. We observed reproducibly high levels of *C. difficile* colonization in mice treated with cefoperazone,

metronidazole, and streptomycin (**Figures 3.1** and **3.2**). We observed variable levels of *C. difficile* colonization in mice treated with ampicillin. None of the mice that received ciprofloxacin were colonized. In addition to administering ciprofloxacin by oral gavage, we provided ciprofloxacin by intraperitoneal injection (10 mg/mL). For both approaches we provided one or two days of recovery. Regardless of the method, the resulting communities were resistant to *C. difficile* colonization. Only one of six mice receiving vancomycin was colonized with *C. difficile.* We suspected that this was due to residual vancomycin repressing *C. difficile* growth. In fact, two days post *C. difficile* challenge, *C. difficile* bloomed in these mice to a median of $9.1x10^7$ (interquartile range $7.6x10^7$–$1.1x10^8$) CFU/g feces. Furthermore, given two days of post-vancomycin recovery, there was no delay in *C. difficile* colonization to high levels, and on day one post challenge we observed a median of $3.0x10^7$ (interquartile range $2.6x10^7$–$3.6x10^7$, N=4) CFU/g feces. These results suggest that although vancomycin is not absorbed by the gut tissue, the absence of *C. difficile* in the remaining five vancomycin-treated mice may have been due to residual antibiotics lingering in the environment. Overall, the various antibiotic perturbations provided varying levels of colonization by *C. difficile,* which suggested that the resulting communities varied in their composition.

To test this hypothesis, we sequenced the 16S rRNA genes from the fecal communities of treated and untreated mice prior to *C. difficile* challenge to identify populations within the microbiota that conferred colonization resistance. All of the antibiotic treatments, except for the ciprofloxacin-treated mice (AMOVA, P=0.09), resulted in distinct and reproducible changes to the structure of the microbiota relative to the untreated animals (AMOVA, P<0.001). Comparisons of the microbiota between

Antibiotic	Administration	Class	Mechanism	Target
Ciprofloxacin	Oral gavage, one time	Fluoroquinolone	Inhibits DNA gyrase	Gram +/-
Clindamycin	Intraperitoneal injection, one time	Lincosamide	Inhibits protein synthesis	Anaerobes
Vancomycin	*Ad libitum* in drinking water, five days	Glycopeptide	Inhibits peptidoglycan synthesis	Gram +
Streptomycin	*Ad libitum* in drinking water, five days	Aminoglycoside	Inhibits protein synthesis	Gram +/-
Cefoperazone	*Ad libitum* in drinking water, five days	β-lactam: Cephalosporin	Inhibits peptidoglycan synthesis	Gram +/-
Ampicillin	*Ad libitum* in drinking water, five days	β-lactam: Penicillin	Inhibits peptidoglycan synthesis	Gram +/-
Metronidazole	*Ad libitum* in drinking water, five days	Nitromidazole	Destabilizes bacterial DNA	Anaerobes

TABLE 3.1 Description of antibiotics used in the study

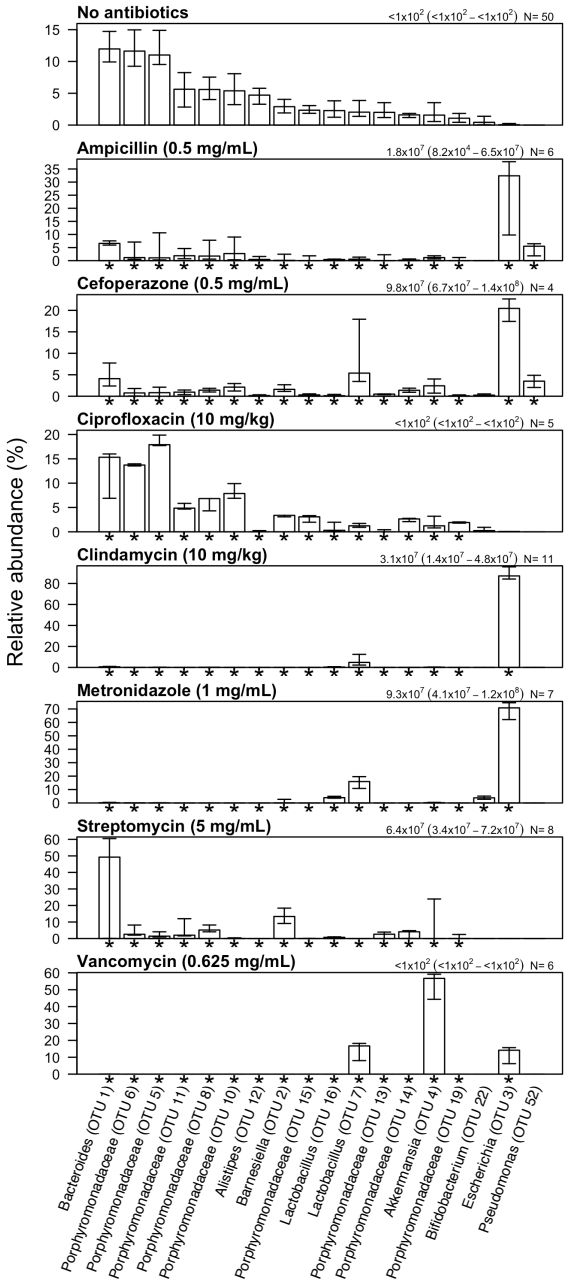

FIGURE 3.1 Antibiotic treatments result in significant alterations to the structure of the microbiota and variation in colonization resistance

Bars indicate the median percent relative abundance of those selected OTUs from all treatment groups on the day of *C. difficile* challenge. Stars along the x-axis indicate those OTUs that were significantly different from untreated mice for that antibiotic treatment. The error bars indicate the interquartile range. The median level *C. difficile* colonization found 24 hours post microbiota sampling and the number of animals in the treatment group is indicated in the top right for each treatment with the interquartile range in parentheses. The concentration next to the name of the antibiotic indicates the dose of the antibiotic that was given to the animals.

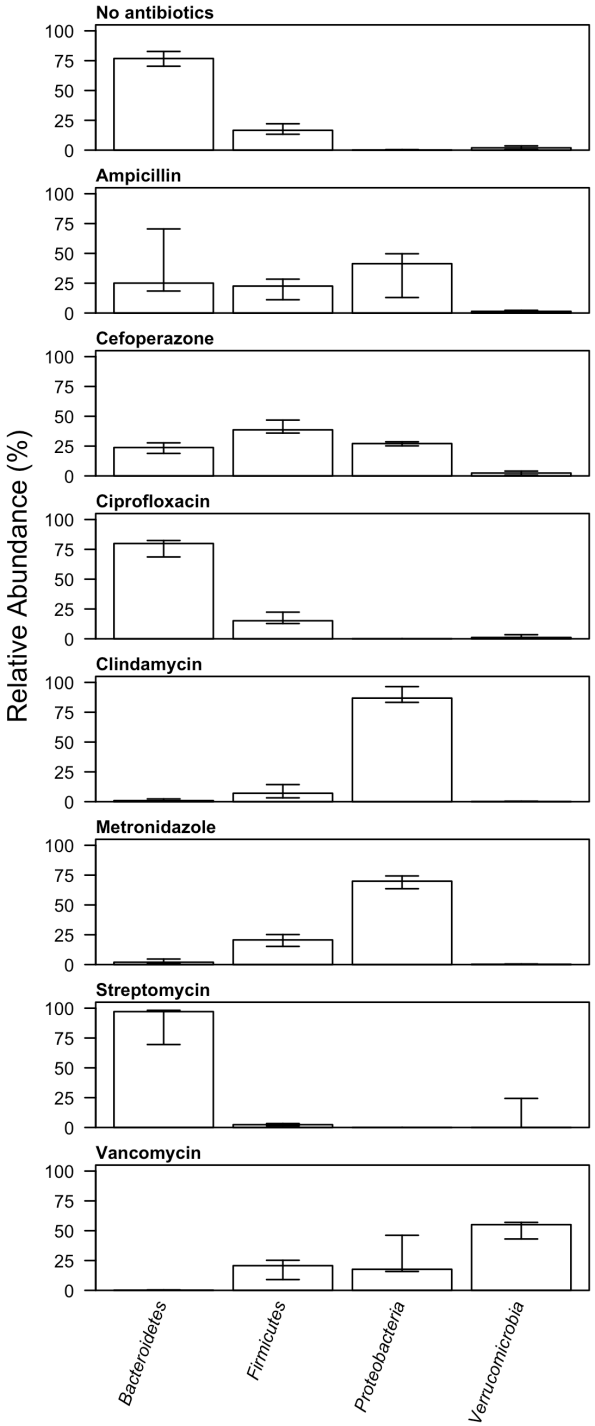

FIGURE 3.2 Effect of antibiotic perturbations on phylum-level representation of communities on day of *C. difficile* challenge
Bars depict the median relative abundance across mice within the treatment group and error bars indicate the interquartile range.

antibiotic classes indicated that their structures were significantly different from each other (AMOVA, P<0.03). The community structures of mice receiving beta-lactams (i.e. cefoperazone and ampicillin) were not significantly different from each other (AMOVA, P=0.37). These results indicate that perturbing the gut microbiota with antibiotics resulted in non-overlapping community structures that yielded significant variation in susceptibility to colonization when challenged with *C. difficile*.

Reduced perturbations result in altered levels of colonization

Based on the *C. difficile* colonization levels in our seven antibiotic treatments, we hypothesized that titrating the dose of antibiotics that the mice received would result in smaller perturbations to the microbiota. Consequently we expected a greater maintenance of resistance against *C. difficile* colonization in these titrated treatment groups. In addition to the previous treatments, we treated mice with lower concentrations of cefoperazone, streptomycin, and vancomycin (**Figure 3.3** and **3.4**). These antibiotics were selected because they are thought to target a broad spectrum of bacteria (i.e. cefoperazone), primarily Gram-negative (i.e. streptomycin), and Gram-positive (i.e. vancomycin) bacteria. As expected in all mice receiving titrated doses of cefoperazone, *C. difficile* colonization levels decreased significantly (P<0.02; **Figure 3.3**). Titrating the dose of cefoperazone in the animals' drinking water resulted in significant decreases in the relative abundance of an OTU associated with the genus *Escherichia* (OTU 3) and a number of rare OTUs. We also observed increases in the relative abundances of OTUs associated with the family Porphyromonadaceae (OTU 5,

10, 11, 13, and 21; **Figure 3.3**). Reducing the dose of streptomycin significantly reduced the colonization levels ($P<0.01$; **Figure 3.3**). Titrating the dose of streptomycin in the drinking water resulted in significant changes in the relative abundance of OTUs associated with the Porphyromonadaceae (OTUs 2, 5, 6, 10, and 11), *Alistipes* (OTU 12), and Bacteroidales (OTU 17). In addition to its anti-Gram-positive activity, vancomycin was also selected because although the community was quite different from untreated mice, we observed high levels of *C. difficile* colonization in only one mouse. We anticipated that lower doses might result in a community structure that would result in colonization. In fact, the 0.3 and 0.1 mg/mL doses of vancomycin resulted in similarly high levels of *C. difficile* colonization ($P=0.96$). Seven OTUs were differentially represented across the three doses of vancomycin. Surprisingly, even though the colonization levels of *C. difficile* did not significantly differ between the mice receiving 0.1 and 0.3 mg/mL of vancomycin in their drinking water, four of the OTUs that had significantly different relative abundances were only found in the lower dose. Three of these were affiliated with members of the Porphyromonadaceae (OTUs 2, 5, and 6) and one was affiliated with a member of the genus *Bacteroides* (OTU 1). Two OTUs affiliated with the *Akkermansia* (OTU 6) and *Lactobacillus* (OTU 8) genera increased with increasing dose and a third OTU affiliated with *Escherichia* (OTU 4) had a mixed response to the dose level. These results suggest that the context in which specific members of the microbiota are found is important in determining the overall resistance to *C. difficile*. For example, the relationship between the *Bacteroides* (OTU 1) and *C. difficile* colonization was positive in streptomycin-treated mice and it was negative in cefoperazone-treated mice. In addition, cefoperazone and streptomycin-treated mice

had high levels of *C. difficile* although the former had significantly higher levels of

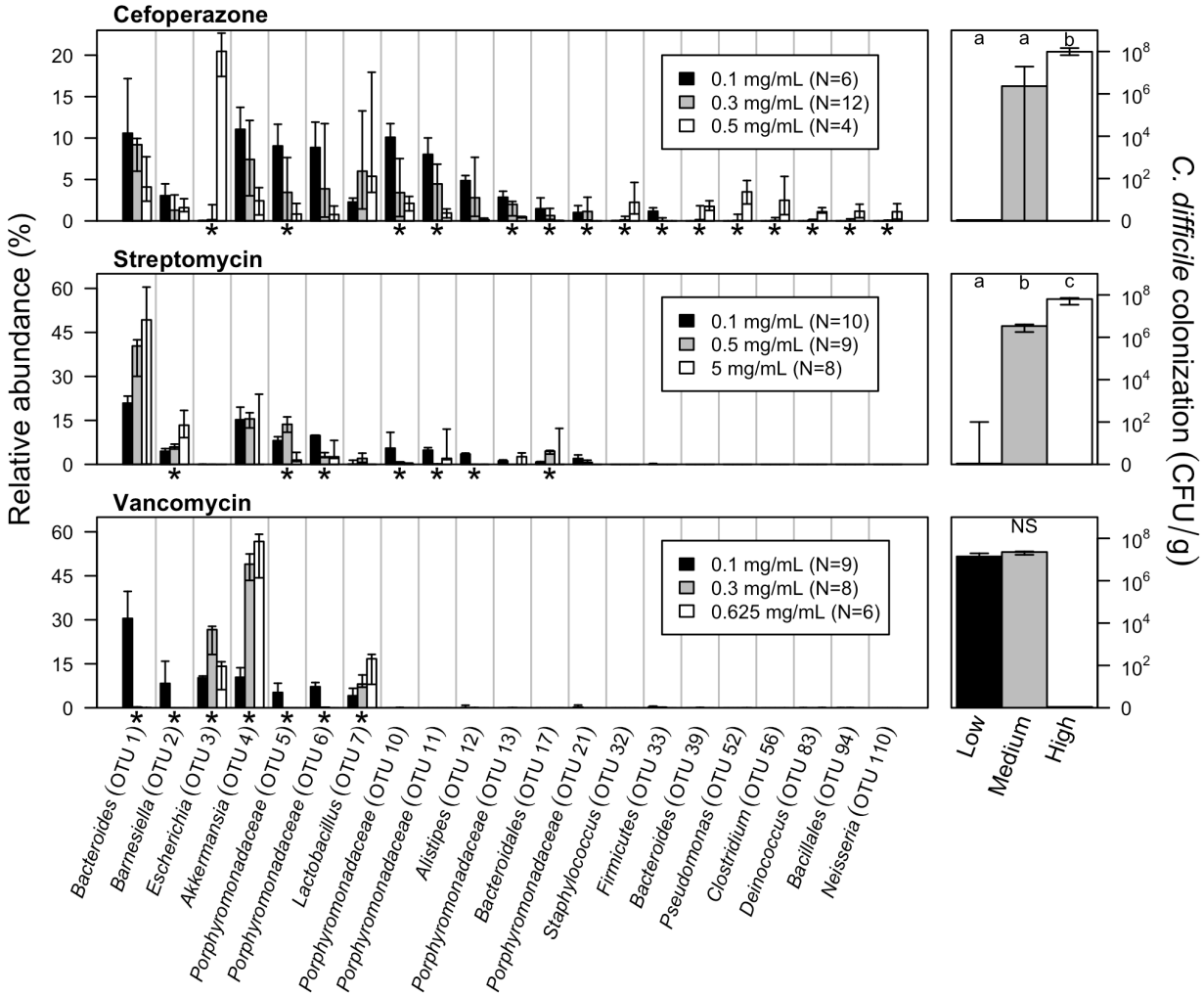

FIGURE 3.3 Titration of antibiotic perturbations results in altered community structures and *C. difficile* colonization resistance

Bars indicate the median percent relative abundance of those selected OTUs from all treatment groups on the day of *C. difficile* challenge. Stars along the x-axis indicate those OTUs that varied significantly across doses of the same antibiotic. The error bars indicate the interquartile range. The median level *C. difficile* colonization found 24 hours post microbiota sampling is plotted on the right for each treatment with error bars indicating the interquartile range. The number of animals used in each treatment group is indicated in the legend, which depicts the doses of each antibiotic that were used.

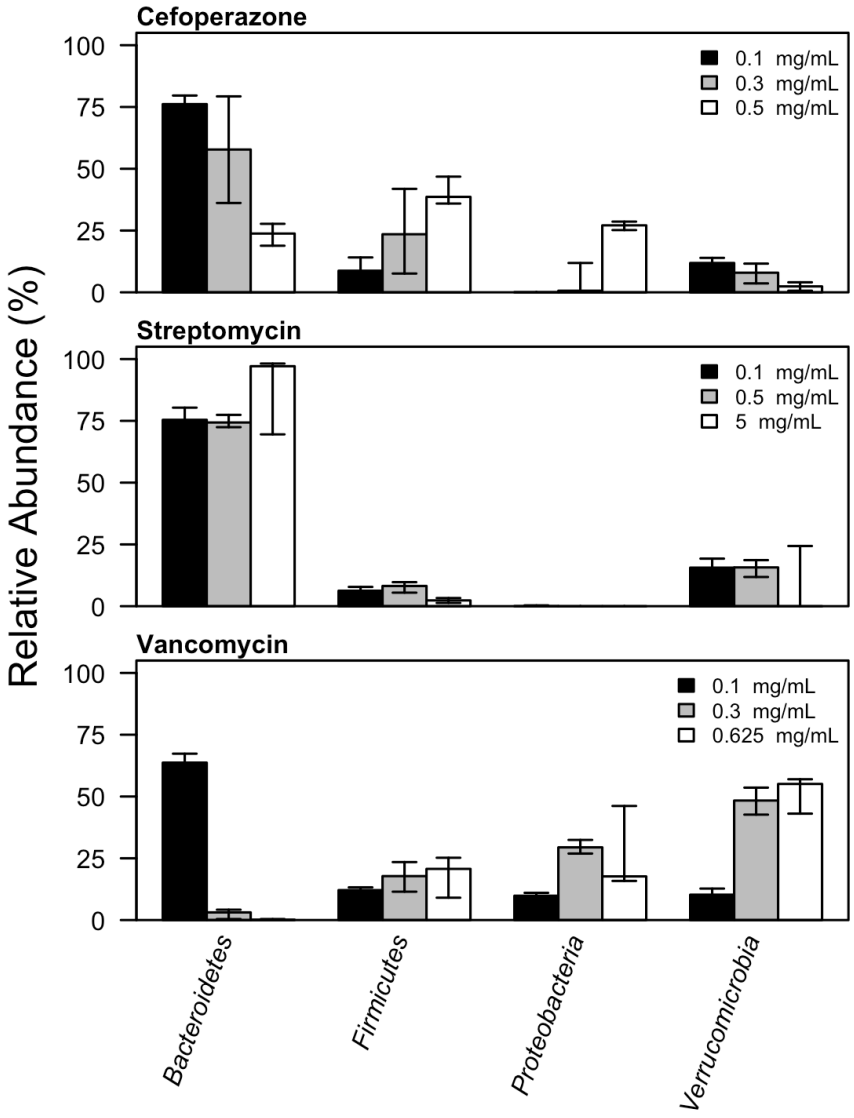

FIGURE 3.4 Effect of titrated antibiotic treatments on phylum-level representation of communities on day of *C. difficile* challenge
Bars depict the median relative abundance across mice within the treatment group and error bars indicate the interquartile range.

Escherichia (OTU 3), which were absent in the streptomycin-treated mice. Together, these results suggest that individual populations were not sufficient to consistently predict colonization resistance. In light of such results, resistance is likely a product of the overall composition of the community.

Allowing recovery of the microbiota restores colonization resistance

In the experiments we have described thus far, we allowed the gut microbiota to recover for 24 hours before challenging them with *C. difficile*. Several studies have demonstrated that perturbed communities can return to a "healthy" state in which resistance to *C. difficile* is restored (3, 8). To test the effect of recovery on colonization and gain greater insights into the populations that confer colonization resistance, we allowed the microbiota of the mice that received the full metronidazole and ampicillin treatment to recover for an additional five days (**Figure 3.5** and **Figure 3.6**). Among the metronidazole-treated mice, those with extended recovery had a 1.8 x 10^6-fold reduction in colonization ($P<0.001$; **Figure 3.5**). In addition, 7 of the 14 mice given the longer recovery period had no detectable *C. difficile* 24 hours after challenge. We detected six OTUs that were differentially represented in the two sets of metronidazole-treated mice (**Figure 3.5**). Most notable among these was a member of the *Barnesiella* (OTU 2) and the *Escherichia* (OTU 3). The relative abundance of this *Barnesiella* OTU increased with the delay, and the relative abundance of this *Escherichia* OTU decreased. Similar to the metronidazole-treated mice, the ampicillin-treated mice that were allowed to recover an additional five days before challenge had a significant

decrease in *C. difficile* colonization (P=0.03). As before, we observed a similar increase

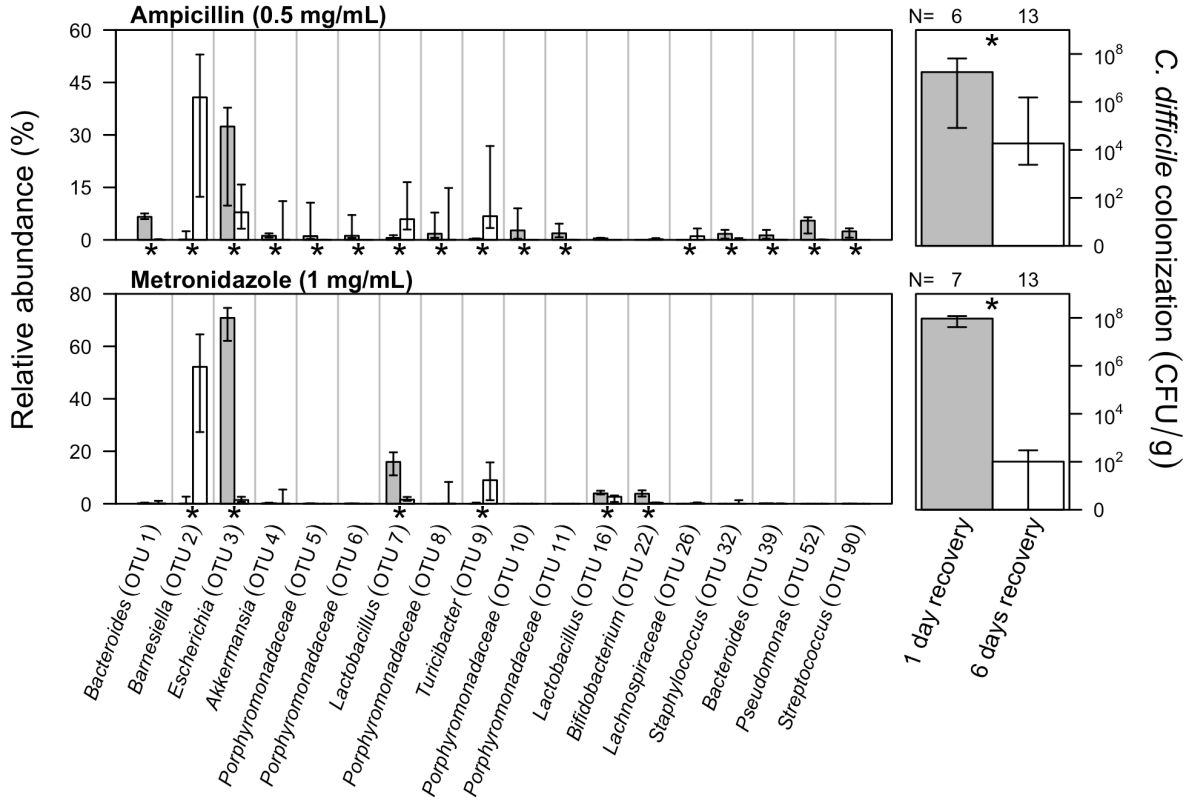

FIGURE 3.5 Increasing the recovery time following antibiotic perturbation restores colonization resistance

Bars indicate the median percent relative abundance of those selected OTUs from all treatment groups on the day of C. difficile challenge. Stars along the x-axis indicate those OTUs that varied significantly between those mice that were allowed 1 or 6 days of recovery. The error bars indicate the interquartile range. The median level C. difficile colonization found 24 hours post microbiota sampling is plotted on the right for each recovery period and antibiotic with error bars indicating the interquartile range. The number of mice used in each treatment group is indicated above the C. difficile colonization data. The dose of each antibiotic is indicated next to the name of the antibiotic.

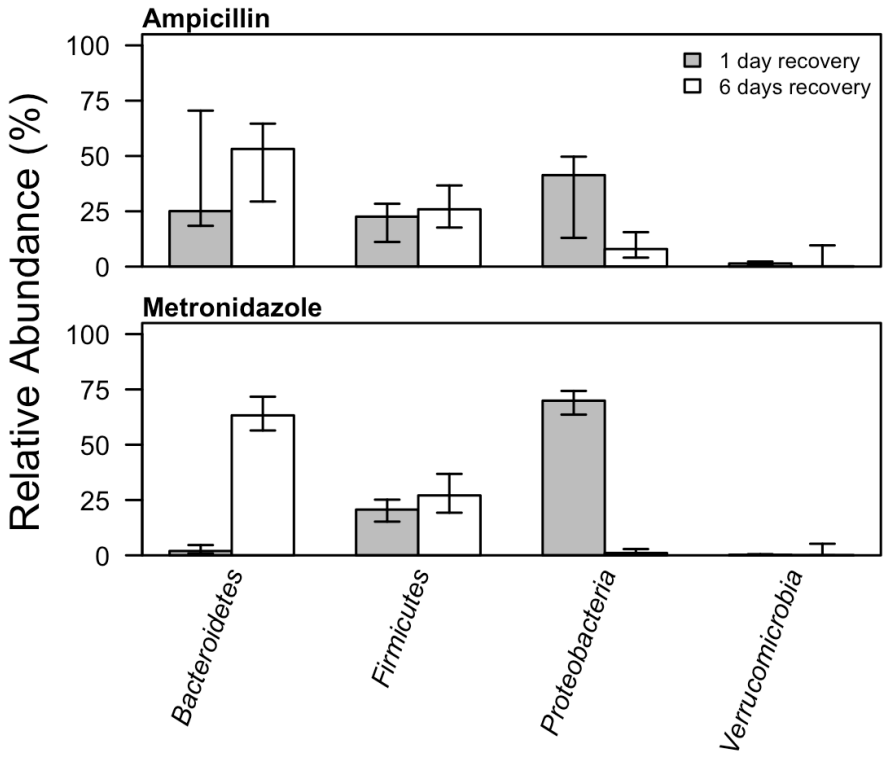

FIGURE 3.6 Effect of recovery period following antibiotic treatments on phylum-level representation of communities on day of *C. difficile* challenge
Bars depict the median relative abundance across mice within the treatment group and error bars indicate the interquartile range.

and decrease in relative abundances for *Barnesiella* (OTU 2) and *Escherichia* (OTU 3). However untreated, fully resistant mice harbored significantly lower levels of *Barnesiella* (OTU 2). Rather, untreated mice had high levels of various Porphyromonadaceae OTUs (**Figure 3.1**). These findings further confirm the context-dependency of colonization resistance suggested by the results of our titration experiments.

Correlation analysis reveals potentially protective bacteria

To identify bacterial taxa that could be associated with resistance or susceptibility to *C. difficile* across the three sets of experiments, we measured the correlation between the relative abundance of each OTU on the day of inoculation with the level of *C. difficile* colonization 24 hours later (**Figure 3.7**). OTUs associated with providing resistance against *C. difficile* (N=22) outnumbered those with associated with susceptibility (N=9). The Porphyromonadaceae ($\rho_{average}$=-0.52, N=11 OTUs) were consistently associated with low levels of *C. difficile* colonization. Among the three Proteobacteria OTUs with a significant positive association with *C. difficile* colonization, the strongest was affiliated with the *Escherichia* (OTU 3; ρ=0.54). By performing an OTU-based analysis we were able to observe intra-family and genus differences in association with *C. difficile* colonization. For example, the Lachnospiraceae have been associated with protection against *C. difficile*. Although we observed three OTUs that were associated with low levels of *C. difficile* colonization, one OTU was associated with high levels of *C. difficile*. In addition we observed three significantly correlated *Lactobacillus* OTUs (family Lactobacillaceae), two of which were associated with low levels of *C. difficile* and one was associated with high levels. The broad taxonomic

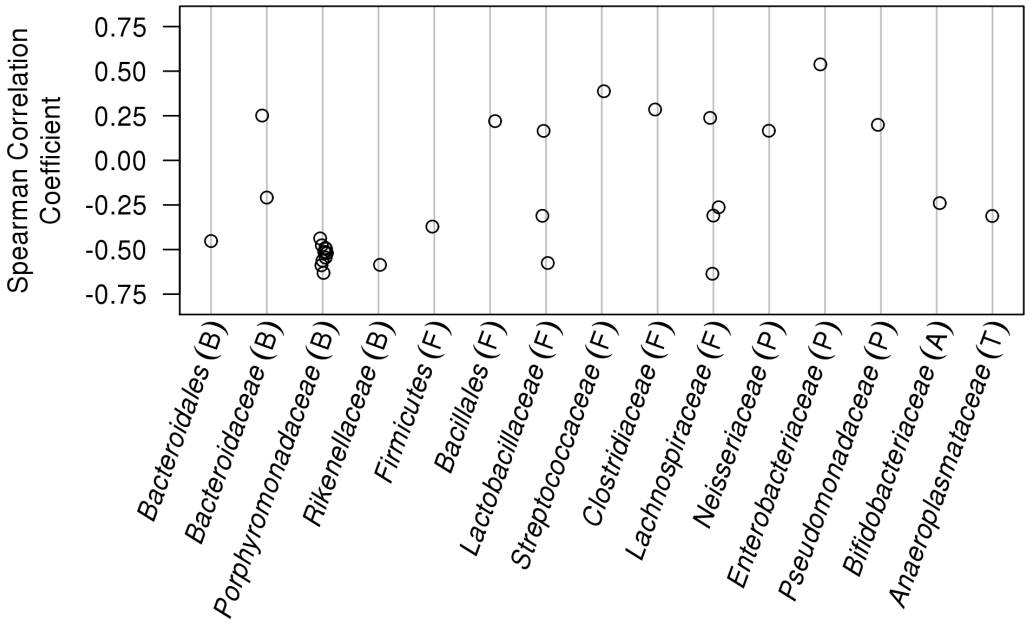

FIGURE 3.7 Diverse taxonomic groups are associated with low levels of *C. difficile* colonization

Spearman correlation coefficients were calculated using the relative abundance of OTUs found on the day that mice were challenged with C. difficile spores and the amount of C. difficile observed 24 hours later. Only significant correlations are presented. OTUs are grouped by the taxonomic family and the letters in the parentheses correspond to the phylum that the taxa belong to. B: Bacteroidetes, F: Firmicutes, P: Proteobacteria, A: Actinobacteria, T: Tenericutes.

representation of OTUs associated with low levels of *C. difficile* again suggests that a functionally diverse community is required to prevent the colonization *C. difficile*.

The composition of the disturbed gut microbiota is predictive of C. difficile colonization levels

These three sets of experiments demonstrated that in certain contexts individual OTUs could be associated with *C. difficile* colonization, but in other contexts those OTUs had the opposite or no association. This suggests that colonization is a phenotype that is driven by multiple populations that act independently and possibly in concert to resist colonization. Correlation-based analyses cannot predict these types of context dependencies because they do not take into account the non-linearity and statistical interactions between populations. Therefore, we used a regression-based random forest machine learning algorithm to predict the level of *C. difficile* colonization observed in the three sets of experiments using the composition of the microbiota at the time of challenge as predictor variables. The model explained 77.2% of the variation in the observed *C. difficile* colonization levels (**Figure 3.8**). When we only included the top 12 OTUs based on the percent increase in the mean squared error when each OTU was removed, the resulting model explained 77.1% of the variation in the observed *C. difficile* colonization levels. The OTUs that were ranked as being the most important in defining the random forest model further validated the observations from the correlation-based analysis (**Figure 3.9**). According to the random forest model, colonization

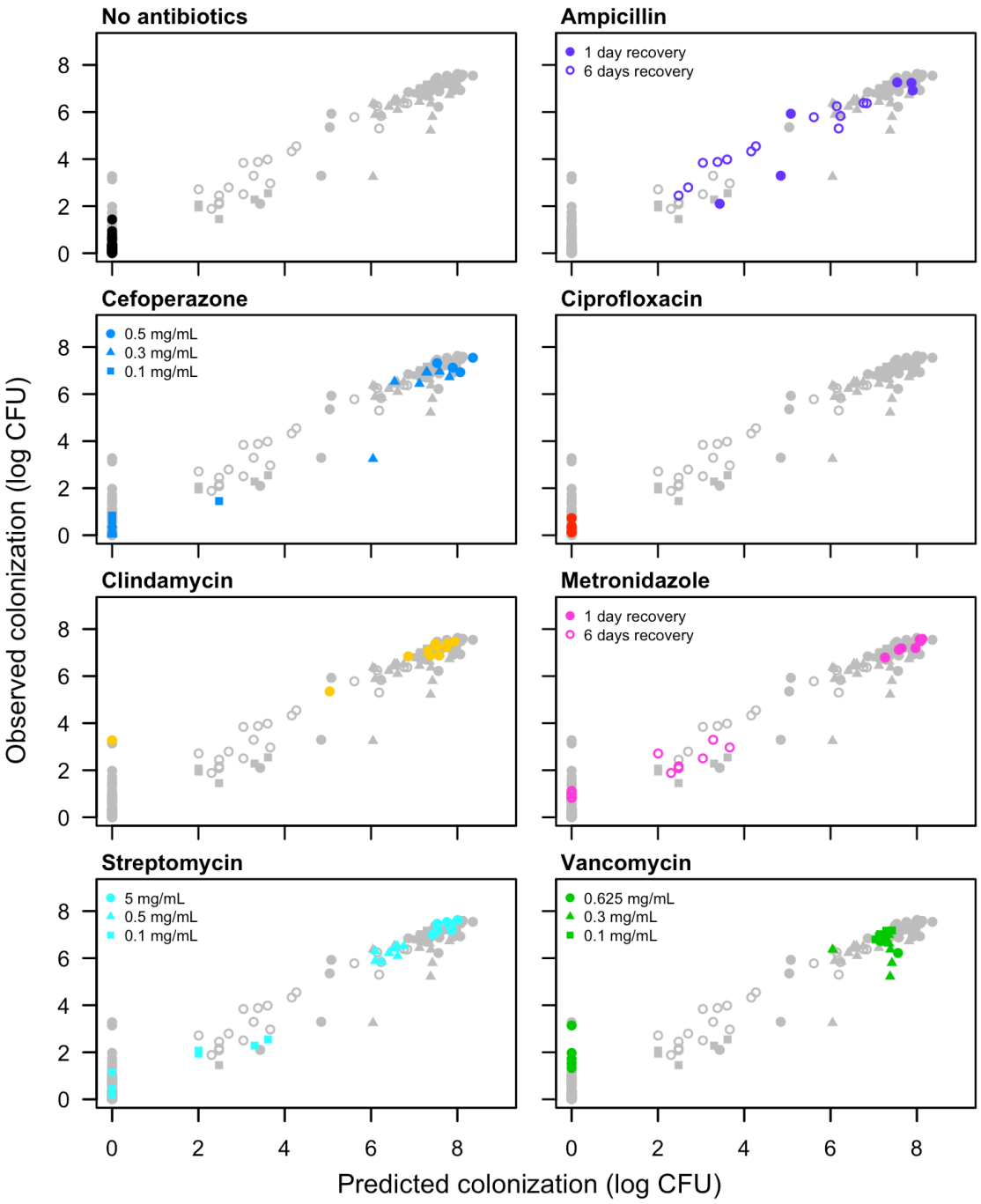

FIGURE 3.8 Random forest regression model predicts *C. difficile* colonization levels based on the structure of the microbiota
The overall model explained 77.2% of the variation in the data. Each pane shows antibiotic treatment groups in color and the other points as gray circles.

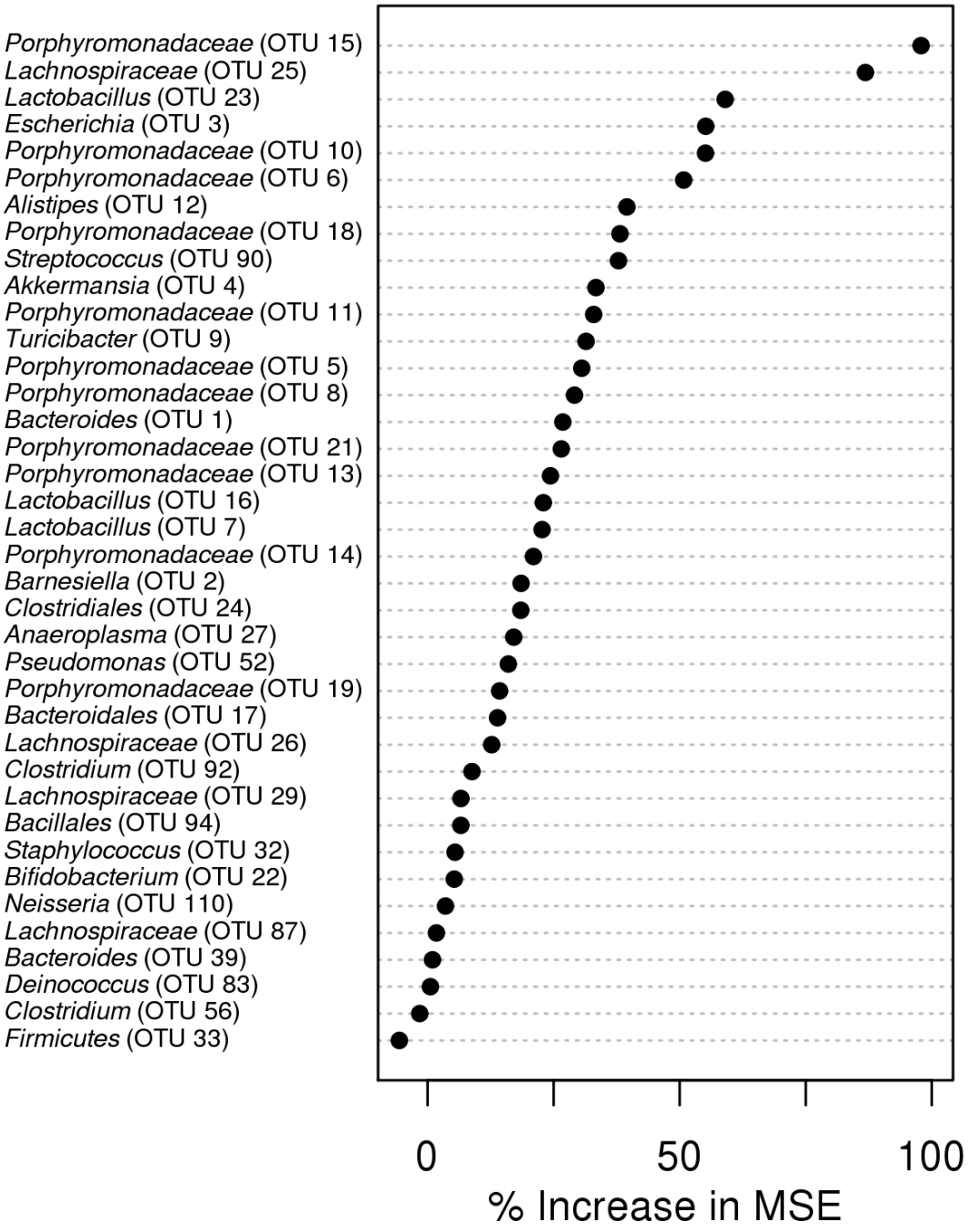

FIGURE 3.9 The change in percent mean squared error when each OTU was removed from the random forest regression model

resistance was associated with members of the Porphyromonadaceae (OTU 15, 10, 6, 18, and 11), Lachnospiraceae (OTU 25), *Lactobacillus* (OTU 23), *Alistipes* (OTU 12), and *Turicibacter* (OTU 9; **Figure 3.10**). A loss in these populations, concurrently with a gain of *Escherichia* (OTU 3) or *Streptococcus* (OTU 90), was associated with an increased susceptibility to infection (**Figure 3.10**). As we observed in the titration experiments, the relationship between *Akkermansia* (OTU 4) and *C. difficile* indicated that wide variation in the relative abundance of *Akkermansia* was associated with varying levels of *C. difficile*. There were varying abundances of *Akkermansia* in mice regardless of the level of *C. difficile* colonization. Finally, as indicated by the number of OTUs with relative abundances below the limit of detection, those mice could harbor varying levels of *C. difficile*. These observations bolster the hypothesis that colonization resistance is context dependent.

Discussion

Previous attempts to study the role of the gut microbiota in colonization resistance against *C. difficile* infection have utilized a single perturbation to the community. Here, we used seven antibiotics from six classes that were given to mice in varying doses and with varying post-antibiotic recovery periods. The result was a combination of 15 different perturbations and the non-perturbed microbiota, which allowed us to generate distinct community profiles that displayed varying susceptibility to *C. difficile* colonization. Our findings demonstrated that colonization resistance was not driven by individual populations, but by a consortium of organisms. Others have demonstrated that *Barnesiella* or Lachnospiraceae are partially protective against *C.*

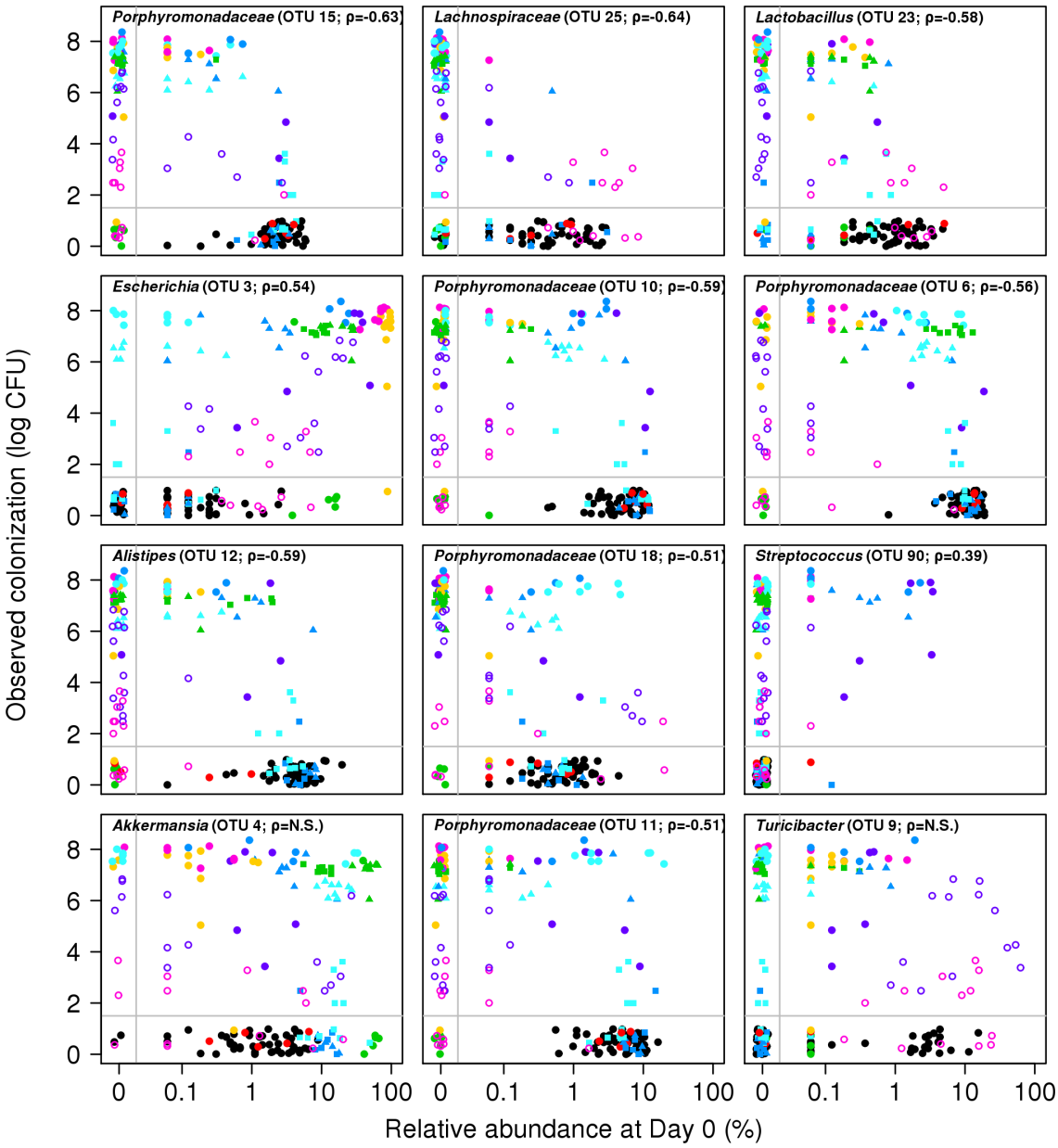

FIGURE 3.10 Relationship between OTU relative abundance and *C. difficile* colonization levels indicates non-linearity and context-dependency
The 12 OTUs that resulted in the greatest change in percent mean squared error when removed from the random forest regression model are shown in each pane and together explain 77.1% of the variation in the data. The Spearman correlation value between that OTUs abundance and C. difficile levels are shown for each pane. The color and symbols represent the same antibiotic dose and recovery period as in Figure 3.8.

difficile (9, 17). Although we observed similar results in a subset of our perturbations, by using a large number of perturbations, we were able to demonstrate that a varied collection of populations was important for complete colonization resistance. Thus colonization resistance can be described as an emergent property of the microbiome, in which individual bacterial populations integrated in a community contribute to the overall resistance to *C. difficile* (18).

There is clear need for more efficient therapies for treatment of *C. difficile* infections in humans aimed at restoration of the microbiota. Current first line treatments of CDI include regimens of either metronidazole or vancomycin, which further perturb the microbiota. As such, relapse rates for CDI are typically around 25-30% (19). Interestingly, we observed that treatment with either antibiotic induced susceptibility to *C. difficile* in mice. This result has implications for understanding the causes of recurrent infections. Previous efforts to restore the microbiota and reestablish colonization resistance also support our findings. For instance, association of germ-free mice with a Lachnospiraceae isolate only reduced the level of *C. difficile* colonization by 10 to 100-fold (17). Using conventional mice, mixtures of bacteria rather than individual populations have been shown to restore colonization resistance and mediate clearance of *C. difficile* (20, 21). Fecal transplants, which represent a diverse collection of bacterial populations, have been highly effective in treating humans with recurrent *C. difficile* (12, 22, 23). By generating a diverse collection of communities that were challenged with *C. difficile*, we have identified a subset of populations using random forest modeling that could be used as a probiotic cocktail to provide colonization resistance. These would

include members of the Porphyromonadaceae, Lachnospiraceae, *Lactobacillus*, and *Alistipes*. Several of these populations have been examined for their potential as a probiotic for preventing *C. difficile* infection. A 6-species cocktail, including isolates of Porphyromonadaceae, Lachnospiraceae, *Lactobacillus*, Coriobacteriaceae, *Staphylococcus*, and *Enterococcus*, successfully resolved CDI in mice (21). In humans, *Lactobacillus*-based probiotics have been co-administered with antibiotics to deter the onset of antibiotic-associated diarrhea (AAD) and *C. difficile* infection (21). A more diverse probiotic, which contained 33 bacterial species including Porphyromonadaceae, Lachnospiraceae, Ruminococcaceae, Eubacteriaceae, and *Lactobacillus* isolates, successfully restored colonization resistance in recurrent *C. difficile* infection and eliminated diarrhea up to 6 months post treatment (24). Given this evidence, we feel confident that an effective probiotic community could be designed based on our findings to recover colonization resistance against *C. difficile*.

Random forest regression models allowed us to describe community resistance as a byproduct of an assemblage of bacterial populations rather than as individual populations. A correlation-based analysis was unable to identify populations that had a context dependent or non-linear association with *C. difficile* colonization. Although the murine and human microbiota do not fully overlap, our previous analysis of humans infected with *C. difficile* supports the populations that we associated with colonization (10). For instance, *Escherichia* was previously associated with infected individuals and Lachnospiraceae, Ruminococcaceae, and *Alistipes* were absent from infected individuals. The overlap between the results from the current study and past human studies along with the power of random forest models suggest that it should be possible

to model a patient's risk of developing a *C. difficile* infection based on their gut microbiota composition at admission. Overall these findings demonstrate the significance of the microbiota as an interconnected bacterial community in assessing resistance to pathogen colonization.

Materials and Methods

Animal care

We used 5-8 week old C57Bl/6 mice for all of our experiments. These mice were reared under SPF conditions within the animal facility at the University of Michigan. All animal-related protocols and experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan and carried out in accordance with the approved guidelines.

Antibiotic administration

Mice were administered one of seven different antibiotics including cefoperazone, vancomycin, metronidazole, streptomycin, ciprofloxacin, ampicillin, and clindamycin (**Table 3.1**). The route of administration depended on the specific antibiotic. Cefoperazone (0.5, 0.3, or 0.1 mg/ml), vancomycin (0.625, 0.3, or 0.1 mg/ml), streptomycin (5, 0.5, or 0.1 mg/ml), metronidazole (0.5 mg/ml), and ampicillin (0.5 mg/ml) were all administered in the mouse drinking water for 5 days. Ciprofloxacin (10 mg/kg) was administered via oral gavage and clindamycin (10 mg/kg) was administered via intraperitoneal injection. Mice that did not receive antibiotics were used as negative controls for these experiments.

C. difficile *preparation and challenge*

All antibiotic-treated mice were given 24 hours to recover with untreated drinking water prior to *C. difficile* challenge. *C. difficile* strain 630Δerm spores were used in all experiments. Spores were prepared from a single large batch whose concentration was determined within the week prior to each *C. difficile* challenge (25). Spores were stored long term at 4°C. On the day of challenge 10^3 *C. difficile* spores were administered to mice via oral gavage. Immediately following this challenge, the remaining *C. difficile* inoculum was diluted in a series and plated to confirm the correct dosage.

Sample collection and plating

Fecal samples were freshly collected for each mouse on the day of *C. difficile* challenge. On the day after the challenge another fecal sample was weighed and diluted under anaerobic conditions with anaerobic PBS. The number of colony forming units (CFU) were counted following 24 hours growth on TCCFA plates at 37°C under anaerobic conditions (26).

DNA extraction and sequencing

Total bacterial DNA was extracted from each stool sample collected prior to challenge using the MOBIO PowerSoil®-htp 96 Well Soil DNA Isolation Kit. We generated amplicons of the V4 region within the 16S rRNA gene and sequenced the fragments using an Illumina MiSeq as previously described (27).

Sequence curation

These sequences were curated using mothur (v.1.35) as previously described (27, 28). Briefly, sequences were binned into operational taxonomic units (OTUs) using a 3% dissimilarity cutoff. Taxonomic assignments were determined by using a naïve Bayesian classifier with the Ribosomal Database Project (RDP) training set (version 10) requiring an 80% bootstrap confidence score (29). In parallel to the fecal samples, we also sequenced a mock community where we knew the true sequence of the 16S rRNA gene sequences. Analysis of the mock community data indicated that the error rate following our curation procedure was 0.02%. All 16S rRNA gene sequence data and metadata are available through the Sequence Read Archive under accession SRP057386.

Statistical analysis and modeling

Complete scripts for regenerating our analysis and this paper are available at the online repository for this study (https://github.com/SchlossLab/Schubert_AbxD01_mBio_2015). Comparisons between the antibiotic-treated communities were made by calculating dissimilarity matrices based on the metric of Yue and Clayton (30). To avoid biases due to uneven sampling, the dissimilarity matrices were calculated by rarefying the samples to 1,625 sequences per sample. We then used analysis of molecular variance (AMOVA) to test for differences in community structure using 10,000 permutations (31). OTU-based analyses were performed using R (v.3.1.2). After subsampling the OTU frequency data

to 1,625 sequences per sample, OTUs were considered for analysis if their average relative abundance within any treatment group was at least 1% (N=38 OTUs). All OTU-by-OTU comparisons were performed using the Kruskal-Wallis rank sum test followed by pairwise Wilcoxon rank sum tests. Comparison of log (base 10) transformed *C. difficile* CFU/g feces between experimental groups was calculated using the Kruskal-Wallis rank sum test followed by pairwise Wilcoxon rank sum tests. Spearman rank correlation analysis was performed between OTU counts and *C. difficile* CFU/g feces. All P-values were corrected using a Benjamini and Hochberg adjustment with an experiment-wide Type I error rate of 0.05 (32). Random forest regression models were constructed using the randomForest R package using 10,000 trees (33). The regression was performed using the log (base 10) transformation of the number of CFU/g fecal material as the dependent variable and the 38 OTUs as predictor variables.

Notes

This work was submitted to *MBio* in April 2015.

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CHAPTER IV

Discussion

The microbiota has been described as a “microbial organ” due to the integral nature of these collective microorganisms in normal host functioning (1). One of the critical functions of this pseudo organ in the gut is to prevent the establishment of and inhibit pathogens’ subversion of the host for its own gain. Both the host and resident microbial community have a common interest in preventing disruption and invasion of the gut habitat by pathogens such as *C. difficile*. To this end, the microbiota acts as the first line of defense against *C. difficile*. The work presented in this dissertation explored the characteristics of resistant and susceptible microbiotas against *C. difficile* colonization.

Chapter Summary

The work described in this dissertation examined the relationships between *C. difficile* and members of the gut microbiota in both humans and mice. Use of statistical models allowed us to verify the contribution of the microbiota in determining *C. difficile* infection status and in predicting *C. difficile* colonization levels. We identified several bacterial populations implicated in resistance or increased susceptibility to *C. difficile*.

Many of these key positive and negative bacteria overlap in both hosts at the family level. These bacteria are potential targets of therapeutic manipulation in order to restore balance and the healthy functions of the microbiota.

Microbiota Markers of C. difficile *Infections*

In Chapter II, the microbiotas of hospitalized patients with and without CDI were characterized. These individuals all had diarrhea, but differed in their clinical testing for *C. difficile*-associated diarrhea. These two groups were further compared to the microbiotas of healthy individuals without diarrhea from the surrounding community. Using logit models to predict *C. difficile* diagnosis, we assessed the contribution of patient microbiota structure data to the performance of a base model incorporating patient epidemiological data alone. The goal of this study was not to improve CDI diagnostics, but rather determine what characteristics of the microbiota defined each group. Microbiota data for the three experimental groups were introduced into the model using three approaches: 1) the inverse Simpson index, a measure of beta diversity of the community, 2) categorical community types, defined through clustering patient microbiotas based on structural distances, 3) the relative abundances of a microbiota subset, identified based on their effect size in our linear determinant analyses (LDA).

Regardless of the method of microbiota incorporation used in distinguishing healthy controls from hospitalized individuals, all microbiota-inclusive models performed as well or significantly better than the base epidemiological model alone. This suggests that there are defining microbiota characteristics between these two populations. For instance, hospitalized individuals, regardless of CDI status, had lower microbiota

diversity compared with community controls. Hospitalized individuals had significant losses in the normally dominant Bacteroidetes and Firmicutes from the control group. These include *Bacteroides*, Ruminococcaceae, *Alistipes*, and Lachnospiraceae many of which have been observed in previous mouse and human studies (**Table 1.1**).

Overall the two hospitalized groups were the most similar with high levels of Enterobacteriaceae and *Enterococcus*. Microbial community types was the only method which significantly enhanced model performance compared with the base model in distinguishing these two groups. Few bacterial species actually distinguished CDI from non-CDI associated diarrhea as determined by LDA, but included other *Clostridium* species, an Erysipelotrichaceae, and a *Blautia* OTU. Low diversity is characteristic of patients with CDI, especially recurrent CDI (2, 3). Because low diversity is also seen in non-CDI associated hospital patients, that both groups had diarrhea is the more probable determinant of their diversity level. It is likely that a community with decreased diversity is advantageous for *C. difficile* colonization. Bacterial community diversity has been recognized as a key factor in resistance to pathogen invasion of soil habitats (4). Specifically, it is proposed that decreased diversity contributes to an advantage in the pathogen's competitive ability for resources (4). Community diversity also contributes to functional redundancy within the community, which helps maintain functional stability and resistance to change (5, 6). Our data suggests that the non-CDI associated diarrhea patients may be susceptible CDI, and factors contributing to diarrhea may in turn shift the microbiota towards a decrease in colonization resistance. Our overall findings emphasize the importance of the microbiota in the context of a community in determining pathogen resistance levels.

Often a major limitation in studying the human microbiota in disease is the lack of individuals' healthy baseline state. Given the high degree of inter-individual variation between the gut microbiotas of humans, cross-sectional human investigations mask this variation at finer taxonomic levels. Murine models of CDI allow for controlled longitudinal experiments to examine the dynamics of the microbiota and *C. difficile* during colonization and infection.

Murine CDI Models Confirm Human Associations

In Chapter III, we used murine models of CDI to measure colonization resistance levels of antibiotic-manipulated microbiotas. By observing CDI in a mouse model we could determine key features of microbiota structures contributing to *C. difficile* resistance. Using a broad representation of antibiotic classes, as well as varying doses and post-antibiotic recovery time, we differentially perturbed the microbiota and examined its effects on *C. difficile* colonization. Across these perturbations, we observed that there was no single bacterial population that was responsible for colonization resistance. Rather, resistance to *C. difficile* is the product of multiple interactions within the community.

Among the seven antibiotics were metronidazole and vancomycin, which are the first choice of antibiotics to treat CDI in humans. Using the highest dose of vancomycin, the subsequent day no *C. difficile* is detected. However by day 2 post challenge (day 3 post antibiotic cessation) there was a huge bloom in *C. difficile* (average 8.5×10^7 CFU/g feces, data not shown). In titrated vancomycin treatment groups, this delay in *C. difficile* bloom was not detected, and *C. difficile* colonized to high levels (approximately 10^7

CFU/g feces), suggesting that residual vancomycin in the highest dose group inhibited *C. difficile* growth on day 1 post challenge. Interestingly, following either vancomycin or metronidazole the resulting bacterial communities are susceptible to *C. difficile* colonization. These results demonstrate the need for alternative first line CDI treatments with the goal of restoring microbiota balance.

We allow partial restoration of the microbiota by delaying *C. difficile* challenge following the termination of metronidazole treatment by five additional days. During that period, metronidazole treated microbiotas showed signs of recovery towards baseline (**Figure 3.5** and **3.6**). Following this extended recovery time, these mice harbored significantly lower *C. difficile* CFU/g feces 24 hours post infection. Similar experiments were performed with ampicillin, in which we also saw a decrease in *C. difficile* colonization following extended post-antibiotic recovery. Both metronidazole and ampicillin delay groups had significant decreases in Enterobacteriaceae and increases in Porphyromonadaceae. Several studies have looked at the required recovery time in humans following antibiotics to allow restoration of microbiota colonization resistance. The first month following antibiotic cessation is associated with an increased risk for CDI, which decreases over the next three months (7-9). These microbiota post-perturbation dynamics differ based on antibiotic and dose (10-14). With recovery time, the diversity of the microbiota increases and colonization resistance is gradually restored as the structure returns to a healthy stable state (11, 13-15). Our delay experiments emphasize the importance of restoring balance to the microbiota for pathogen resistance.

By applying a variety of antibiotic types and degrees of perturbations, we collected data on microbiota structures with a range of colonization resistance levels. We show that a regression-based random forest model built using these microbiota relative abundances accurately predicts *C. difficile* levels the subsequent day (**Figure 3.8**). Species-level OTUs with the most contribution to the model's accuracy corroborate the importance of several Bacteroidetes and Firmicutes previously associated with resistance against *C. difficile* in both mice and humans (**Table 1.1**, **Figure 3.9**). Interestingly, Porphyromonadaceae was found in healthy mice, whereas in Chapter II we show that *Bacteroides* were the predominant Bacteroidales population associated with health in humans. This suggests there are host-specific structures involved in colonization resistance.

Turicibacter and *Akkermansia* are among the top 12 OTUs contributing to accuracy of our model, and yet their correlations with subsequent C. difficile colonization levels are not significant. *Turicibacter* was largely found in microbiota of mice given 5 additional recovery days prior to *C. difficile* challenge, in which we observed decreased *C. difficile* colonization. *Akkermansia* significantly blooms in the microbiota of mice treated with vancomycin, which induced high levels of *C. difficile* colonization. Interestingly, across all antibiotic treatments this OTU was not correlated with subsequent *C. difficile* levels. Both the low dose streptomycin and cefoperazone treatments (0.1 mg/mL) also had high levels of *Akkermansia* but resulted in low *C. difficile* levels 24 hours later. These two groups with higher colonization resistance also had no Enterobacteriaceae (OTU 4) and little to no overall Proteobacteria. These findings suggest that colonization resistance is context dependent. Of note,

Akkermansia is a known host mucin degrader. While *C. difficile* is able to utilize glycans in host mucin, it does not contain the necessary enzymes to free these resources (16). High levels of *Akkermansia* may contribute to available nutrients for *C. difficile* growth. Degradation of the mucus layer by high levels of *Akkermansia* contributes to increased inflammation and susceptibility to GI infection by *Salmonella enterica* Typhimurium (17). Increased inflammation is favorable for Enterobacteriaceae such as *E. coli*, which can utilize the nitrates generated during inflammation (18, 19). Thus, it is possible that the importance of *Akkermansia* or *Turicibacter* is in its interactions with other bacteria with stronger positive or negative relationships with *C. difficile*, which would account for the lack of direct correlation between these bacteria and *C. difficile*.

The research presented in this dissertation outlines key bacterial groups implicated in *C. difficile* resistance. Use of statistical models based on bacterial abundances accurately predicts *C. difficile*'s ability to colonize, effectively measuring the level of colonization resistance of the microbiota. These findings have clinical applications in CDI risk assessment based on gut microbial markers. The protective bacterial groups identified in these studies should be further tested for their efficacy as probiotics that restore *C. difficile* resistance.

Future Directions

Expanding a Microbiota Model of CDI

Mathematical modeling is a powerful tool for uncovering interactions among variables (20-22), predicting responses to perturbation (23-25), and assessing biomarkers and risk of disease (26, 27). Future studies expanding the degrees and

types of perturbations and their subsequent effects on colonization resistance will refine the model and identify a broadly encompassing model. Extending the titration studies in Chapter III, I propose modifying or adding titration groups to cover the entire range from no colonization to maximal *C. difficile* colonization for each antibiotic. These studies may indicate if an abundance threshold exists for protective bacteria, either collectively or for individual protective populations. Furthermore perturbations other than antibiotics will likely have unique effects on the microbiota, host, and subsequent susceptibility to *C. difficile*. In a hospital setting several factors can potentially affect patients' microbiota structure, including diet (28, 29), an array of medications (e.g. antibiotics (10, 11, 13), proton pump inhibitors (30), or laxatives (31)), chemotherapy (32, 33), and comorbidities (34-36). Inclusion of this expansive dataset can help improve our model. Given the ability of models to accurately determine disease status and predict pathogen colonization, there may also be potential in using markers of the microbiota to assess risk for CDI and predict disease severity.

Direct Assessment of Bacteria in Colonization Resistance

This dissertation has presented strong evidence for several bacteria in colonization resistance against *C. difficile.* Follow up studies should assess the direct contribution of these bacteria in germfree mice. In Chapter II, microbiota association studies in humans suggested the importance of *Bacteroides*, Ruminococcaceae, *Alistipes*, and Lachnospiraceae in *C. difficile* resistance. In Chapter III, controlled murine models of CDI support these findings in addition to highlighting Porphyromonadaceae's

protective influence in mice. I hypothesize that effective tailored probiotic CDI treatments can be designed using various combinations of these bacteria.

There is evidence for the success of probiotics in treating CDI. Used as a prophylactic concurrently with antibiotics, probiotics have been shown to significantly reduce the risk of antibiotic-associated diarrhea (relative risk = 0.61) and CDI (relative risk = 0.37) in a meta-analysis of 16 studies (37). A 33-species probiotic was tested for its ability to resolve recurrent CDI in two patients (38). These species were isolated from a healthy adult donor and screened to ensure sensitivity to a range of antibiotics. This community probiotic was designed to reflect bacterial abundances found in previously characterized healthy human guts. A 100mL treatment (estimated concentration=3.5 x 10^9 colony-forming units/ml) successfully cleared these individuals of CDI and relieved their diarrhea, even with incidental antibiotic treatments in subsequent weeks. Day 2 and week 2 post treatment, these patients had increases in Bacteroidaceae, Porphyromonadaceae, Bifidobacteriaceae, Lachnospiraceae, Clostridiales, and Verrucomicrobiaceae with reductions in Enterobacteriaceae. These results reflect the relationships observed in Chapters II and III of this dissertation.

In the clindamycin-induced murine model of CDI, a probiotic community of six bacterial species successfully restores the microbiota and clears *C. difficile* (39). These were isolated from healthy feces passaged overnight to enrich for cultivable probiotic candidates. Three of the bacteria in their protective mix were identified in Chapters II and III as having protective roles, including an *Anaerostipes* sp. nov. (a Lachnospiraceae), a *Bacteroidetes* sp. nov. (a Porphyromonadaceae), and a *Lactobacillus reuteri* (a Lactobacillaceae). Only these three species of the six were

found in all mice on all days subsequent to treatment. Interestingly, the Porphyromonadaceae isolate was present in the probiotic at 3% of total clones tested, which bloomed to as high as 26% by day 4 post treatment. The remaining three species, *Enterococcus hirae*, *Staphylococcus warneri*, and *Enterorhabdus* sp. nov., were present in low abundance or not at all 4-14 days post-treatment. Both *Enterococcus* and *Staphylococcus* were positively associated with *C. difficile* colonization in Chapter III, although this was not significant (data not shown). *Enterococcus,* however, was observed in Chapter II at high levels in hospitalized individuals, including CDI cases (**Figure 2.4**). The work presented in this dissertation provides new insights into the relationships between microbiota members and *C. difficile,* which will allow for more informed designs of community probiotics.

To design and test these probiotics, these bacteria should ideally be isolated from mice to test their resistance against *C. difficile* in germfree mice. It has been demonstrated that proper immune function occurs in germfree mice only when transplanted with host-specific bacteria (40). However, commercially available strains can also be used for difficult species. Selecting from our pool of candidate protective bacteria, monocolonization studies can be used to assess the individual contribution of each bacterial species in resistance to *C. difficile* colonization (41). However, this is not the context in which these bacteria confer resistance under normal circumstances. Another approach would be to use conventional mouse models of CDI to test the efficacy of designed community probiotics. Using this system, we can test the ability of the probiotics to induce a shift in dysbiotic microbiotas towards health. I would administer small communities that reflect their normal proportion in the mouse

microbiota as performed previously (38), immediately following the cessation of antibiotics. These mice would then be challenged with *C. difficile* to test the resistance of the resultant community. In order to ensure establishment of probiotic species, probiotic boosts should also be tested for their contribution to increasing treatment effectiveness. Because we know how various antibiotics alter the community structure from Chapter III, we can test if the same probiotic combination works differently or similarly in all antibiotic-perturbed backgrounds. Given some of the differences in potentially protective bacteria between mice and humans, similar studies can be performed using bioreactors seeded by human intestinal microbiota. This research could lead towards a better understanding of microbiota dynamics and lead towards methodologies in personalized probiotics.

The Microbiota's Role in "Infection" Resistance

Given the microbiota's heavy hand in resisting *C. difficile* colonization, it is likely that the gut microbiota also plays a role in limiting *C. difficile*'s infection. The pathology observed during CDI is driven by *C. difficile's* two primary toxins, A and B. I hypothesize that the microbiota contributes to infection resistance against *C. difficile* by negatively regulating toxin production. Several environmental signals have been shown to regulate toxin expression and production. Cysteine, proline, an amino acid mixture, lactic acid, butanol, and biotin have demonstrated negative effects on toxin expression and production (42-47). Recently it has been demonstrated that a thiolactone quorum signaling peptide regulates *C. difficile* toxin production. Accumulation of thiolactone produced by *C. difficile* stimulates toxin production. Given the microbiota's influence on

gut environmental signals, it may be possible for microbiota members to interrupt or block this pathway.

We know that using the cefoperazone-induced murine model of CDI, *C. difficile* strain VPI 10463 begins producing its toxins between 18-24 hours post infection (48). To test the effect of other bacteria on toxin production, I propose administering the individual or small community probiotics examined above for their contribution to colonization resistance. These would be orally gavaged at 18 hours post infection, which is prior to the expected appearance of toxin in the cecum, colon, and stool (48). At 24 hours post infection, the toxin levels in these sites would be quantified. Because the microbiota has a known role in mediating pathogen clearance in *Salmonella enterica* serovar *Typhimurium* infections (49), quantifying *C. difficile* levels will distinguish decreases in toxin induced by probiotics from decreases simply due to pathogen clearance. Timing of treatment may be important as well. It could be possible the probiotic should be administered in an earlier window to allow potential changes to occur before the expected onset of toxin production and sporulation. Healthy mouse cecal or colon content should be included as a positive control group in limiting toxin. The influence of environmental nutrients on both *C. difficile*'s growth, toxin production gives importance to the microbiota's effect on available resources. This highlights the host-protective abilities of the microbiota throughout *C. difficile*'s colonization and infection process.

Ecological Model of *C. difficile* Colonization

Across our characterization of susceptible and resistant microbiotas in both mice and humans, several common factors led us to the ecological model in **Figure 4.1**. Upon an external perturbation, depending on the type and magnitude, the microbiota is altered to a certain degree. If this perturbation is significant, it can lead to a loss in diversity and contribute to overall instability of the microbiota. The structure of the bacterial community in a susceptible state in both mice and humans is associated with increases in various Proteobacteria, notably *Escherichia* and *Pseudomonas*, as well as *Enterococcus,* and members of the Lactobacillaceae. We suspect that *Akkermansia*'s contribution to *C. difficile* colonization is context-dependent on the presence of other bacteria. For example, in the presence of *Escherichia*, *Akkermansia* may aid in colonization of *C. difficile* by providing mucin-stored nutrients and subsequently inducing increases in inflammation. This can further support the growth of inflammatory bacteria, including *E. coli*. These bacteria not only have decreased competitive ability against *C. difficile*, but they also potentially contribute to creating an environment suitable for colonization and growth.

Less severe perturbations to the microbiota may alter the community structure but not induce dramatic shifts from the healthy baseline community or sufficiently decrease overall diversity. In these microbiota structures we observe higher levels of colonization resistance against *C. difficile.* These communities are characterized by high levels of Bacteroidetes overall and Firmicutes, specifically various Lachnospiraceae populations, Ruminococcaceae, and members of the Lactobacillaceae. Different

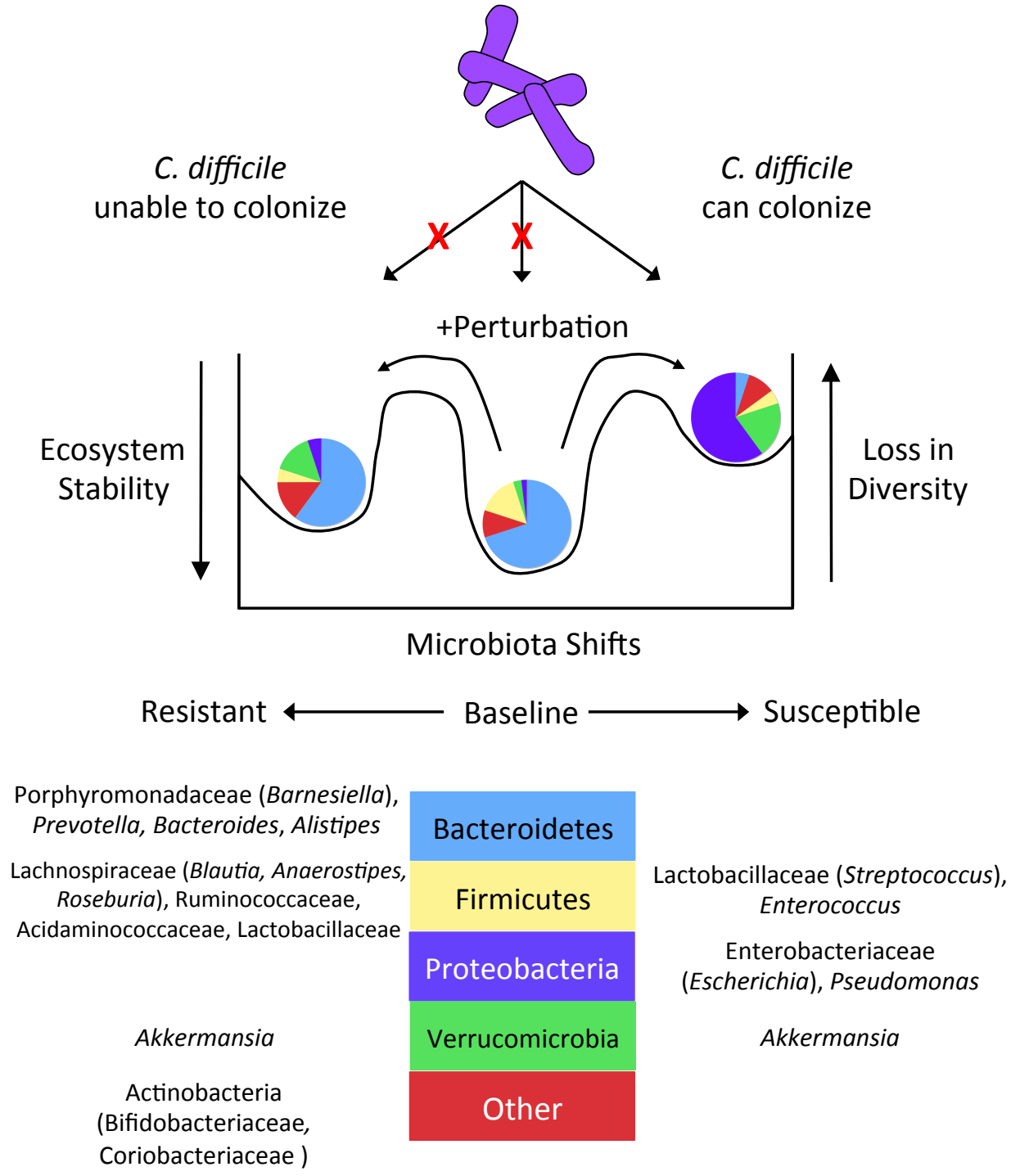

FIGURE 4.1 Ecological model of *C. difficile* colonization

consortia of these bacteria can be protective against *C. difficile,* and together contribute to the overall level of colonization resistance against this pathogen.

Final Conclusions

The gut microbiota plays a crucial role in colonization resistance against *C. difficile*. The findings of this work through both microbiota perturbation studies and mathematical modeling in mice and humans define a collection of bacterial populations from which to efficiently design probiotic therapies for CDI. Traditional antibiotic therapeutics eliminate *C. difficile* but leave collateral damage to the gut microbiota. *C. difficile* treatments aimed at restoration of a healthy-like microbiota, such as FMTs and probiotic cocktails, not only help mediate clearance of *C. difficile*, but also reestablish colonization resistance against future infection. Furthermore, this work could lead to better risk assessment for CDI in health care-associated settings. Patients at high risk may be given probiotics to prevent CDI altogether. Given the high degree of inter-individual variation among healthy microbiotas, it may also be beneficial to personalize treatments based on an individual's particular microbial needs. This work enhances our understanding of key bacterial populations involved in resistance or susceptibility to *C. difficile*, making personalized microbiota treatments against *C. difficile* and potentially other gastrointestinal pathogens a real possibility.

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